LYMPHOCYTE RECEPTORS FOR GLYCOSAMINOGLYCANS

by

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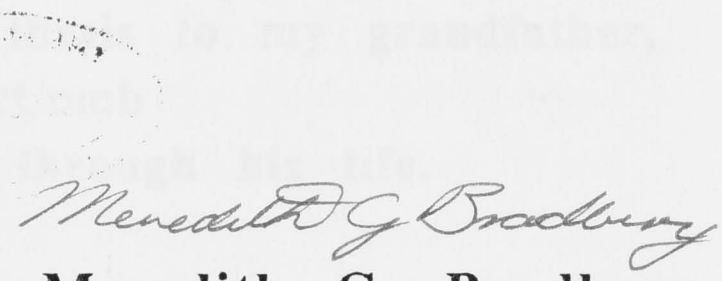
STATEMENT

I certify that the work described in this thesis is my own, unless where stated otherwise, and has not previously been submitted for a degree at this or any other University.



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DEDICATION

I would like to dedicate this thesis to my grandfather,
Mr V.S. McComb
who has inspired me through his life.

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ABSTRACT

Previous studies have shown that lymphocytes carry cell surface receptors for SPS and SPS recognition may play a role in lymphocyte migration and positioning *in vivo*. This thesis describes attempts to isolate and characterise endogenous GAGs and GAG-binding molecules of murine spleen and determine if these molecules are involved in lymphocyte migration.

In Chapter 3 a procedure was devised for isolating GAGs from murine spleen in good yield and high purity and the GAG preparation was then radiolabelled for subsequent binding studies. It was found that endogenous heparan sulfate/heparin-like molecules (MW approx. 50 kDa) bound to murine splenocytes in a saturable, rapid and reversible manner with a binding affinity of approx. 5×10^{-8} M. Using immunofluorescent flow cytometry studies it was demonstrated that the majority of spleen cells have receptors for these GAGs.

Subsequent, binding studies with radiolabelled GAGs (Chapter 4) demonstrated that bovine lung heparin has a binding affinity. to splenocytes of approx. 1.1×10^{-6} M, which is approx. a 20 fold lower affinity than that observed with the endogenous splenic heparin/heparan sulfate molecules. However, due to the large amount of non-specific binding incurred in this assay the binding affinities for other GAG molecules could not be calculated.

Rosetting studies with GAG-coupled red cells demonstrated that between 20% and 90% of lymphocytes express receptors for all the GAGs tested. Analysis of the binding of solubilised radiolabelled cell surface molecules to immobilised GAGs revealed that murine lymphocytes express at least 12 distinct cell surface receptors for GAGs (molecular masses of 10-250 kDa). Each GAG bound to a unique profile of cell surface molecules, although splenocytes exhibited a much more heterogeneous binding pattern than thymocytes. Immunoprecipitation studies demonstrated that the GAG-binding molecules on splenocytes did not correspond to

any of the cell surface antigens tested, namely MEL-14, FcR, CD3, ThB, Ly-5, Ly-15, Pgp-1 and Thy-1, although some data suggested that Ly-2 may bind weakly to heparin (Chapter 4).

Characterisation of a very prominent 90 kDa GAG-binding molecule (Chapter 5) on lymphocytes revealed that it is a peripheral membrane glycoprotein with a pI of 6.16. High ionic strength (2 M KCl) treatment released the molecule from cells although RGDS (1 mg/mL) treatment did not, suggesting an integrin binding sequence was not involved. Subsequent studies suggested that the molecule is bound to the lymphocyte surface via a PI receptor as (i) inositol hexaphosphate displaced the 90 kDa protein from the splenocyte surface and other inositol derivatives were either ineffective or much less effective at displacing the molecule and (ii) reassociation experiments demonstrated that the soluble 90 kDa protein reassociated with lymphocytes in an inositol hexaphosphate inhibitable manner. Additional studies are required to unequivocally demonstrate the existence of a PI receptor on lymphocytes. Such a receptor has important implications for GPI-anchored cell surface molecules.

Attempts were made to relate the expression of GAG-binding molecules to the splenic and lymph node homing capacity of lymphomyeloid cell lines. It was found that certain cell lines failed to enter the spleen (EL-4, BL/VL3, MBL-2, RK4.7, C6VL/1, P815 and LSTRA) while others gained access (BCL.1, $RD10_S$, $R1^+$, $R1^-$ and CL2-FT2). Positioning of fluorescently-labelled cell lines in the spleen was visualised by fluorescence microscopy and revealed that certain cell lines ($R1^+$, BCL.1 and $RD10_S$) migrated into the red pulp and marginal zones of the spleen. Entry of the cell lines into other lymphoid organs and the liver and lungs, was found to be unrelated to splenic entry.

SDS-PAGE analysis of ^{125}I-labelled cell surface molecules indicates that each cell line examined (EL-4, $R1^+$, BCL.1 and $RD10_S$) expressed distinct GAG-binding molecules with a wide range of molecular masses. Interestingly the 90 kDa GAG-binding molecule on lymphocytes is expressed strongly by both $R1^+$ and BCL.1, but

not by $RD10_S$ indicating that this protein is not necessary for splenic entry. However, an approx. 35 kDa GAG-binding protein is present on all three cell lines which migrated to the spleen ($R1^+$, BCL.1 and $RD10_S$) but is absent from EL-4, a cell line which did not enter spleen. Further experiments are required to prove that this molecule is involved in splenic-homing.

In conclusion, data are presented in this thesis which are consistent with the view that GAG recognition by lymphocytes may control the entry and positioning of lymphocytes within lymphoid organs. However, further work is required to conclusively demonstrate the role of the various GAG receptors identified in lymphocyte migration.

CONTENTS

ABBREVIATIONS

BSA	bovine serum albumin
C	constant
CMC	carboxymethyl cellulose
Con A	concanavalin A
cpm	counts per minute
CSF	colony stimulating factor
DDW	double distilled water
ECM	extracellular matrix
ECMRIII	extracellular matrix receptor type III
EGF	epidermal growth factor
ELAM-1	endothelial leukocyte adhesion molecule 1
F15	Eagle's minimum essential medium
FB	fibrinogen
FCS	foetal calf serum
FN	fibronectin
F1P	fructose-1-phosphate
GAG	glycosaminoglycan
GALT	gut associated lymphoid tissue
GMP	granule-membrane protein
GPI	glycosyl-phosphatidylinositol
HEBF	high endothelial binding factor
HEC	high endothelial cells
HEV	high endothelial venules
HS	inositol hexasulfate
ICAM	intracellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
LAM1	leukocyte adhesion molecule 1
LFA-1	lymphocyte function-associated antigen-1
LPAM-1	lymphocyte PP HEV adhesion molecule-1
mAb	monoclonal antibody
MALT	mucosal associated lymphoid tissue
MHC	major histocompatibility complex
MLN	mesenteric lymph node
1-MP	inositol-1-monophosphate

2-MP	inositol-2-monophosphate
M6P	mannose-6-phosphate
MW	molecular weight
NCAM	neural cell adhesion molecule
PALS	periarteriolar lymphoid sheath
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
pI	isoelectric point
PI	phosphatidylinositol
PI-PLC	phosphatidylinositol-specific phospholipase C
PP	Peyer's patch
PPME	phosphomannan ester
PS-coupled	polysaccharide-coupled
RGD	arginine-glycine-aspartic acid
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPS	sulfated polysaccharides
SRBC	sheep red blood cells
TCA	trichloroacetic acid
TcR	T cell receptor
TGF-β	transforming growth factors β
TS	0.15 M NaCl, 0.05 M Tris-HCl pH 8.0
TTS	0.15 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.5% Triton X-100
V	variable
VLA	very late antigens
VN	vitronectin
VWF	von Willebrand factor

CHAPTER 1 : INTRODUCTION

1.1 INTRODUCTION

Lymphocytes are in a constant state of flux, recirculating between the bloodstream and lymphoid tissue (Gowans and Knight, 1964), a process which allows maximal exposure of a captured antigen in a particular lymphoid organ to migrating lymphocytes (reviewed by Kieran *et al.*, 1989; Yednock and Rosen, 1989). A major entry point of blood-borne lymphocytes into lymphoid organs is through specialised postcapillary venules, termed high endothelial venules (HEV). This process of lymphocyte extravasation is mediated by the highly selective interaction of cell surface molecules on the lymphocyte and HEV. Alternatively lymphocytes may gain access to a secondary lymphoid organ, such as the spleen, directly via the bloodstream without HEV binding. Neither of these processes are well understood. However, the recent upsurge in molecular technology has led to an increasing awareness of the important role of specific cell surface molecules in these interactions.

The positioning of lymphocytes within lymphoid organs is also highly selective, with subsets of lymphocytes migrating to discrete anatomical locations within the lymphoid organ. Indeed lymphocyte recirculation and positioning within organs is a highly specialised process as implied by the term "homing" (Gowans and Knight, 1964), used to describe the migratory preference of a lymphocyte for a particular lymph node site. The vital role that carbohydrate recognition plays in this system has only recently been realised and represents a major research theme of this thesis.

1.2 STRUCTURE OF THE LYMPHOID SYSTEM

The lymphoid system is composed of usually mobile lymphocytes and sessile epithelial and stromal cells arranged either as discrete organs or accumulations of diffuse tissue. Lymphoid organs may be classified as either primary or secondary organs depending on certain criteria as outlined below.

1.2.1 PRIMARY LYMPHOID ORGANS

Primary lymphoid organs are the major sites of lymphopoiesis in the body, consisting of the thymus for T cells and the bone marrow (mammals) or the bursa of Fabricus (avian species) for B cells. Here lymphocytes acquire specific antigen receptors with the ability to discriminate between self and non-self antigens.

The bone marrow which constitutes 4.5% of the body weight has a large pool of haemopoietic stem cells which are capable of differentiating into various blood cell components such as lymphocytes, mononuclear phagocytes, granulocytes, platelets and erythrocytes. It can be divided into the active, haemopoietic material and the inactive, fatty marrow which consists of a framework of stroma intimately connected with the blood vessels. The stroma consists of primitive and phagocytic reticular cells attached to argyrophilic fibres. Stromal cells produce regulatory substances including CSF (colony stimulating factor), IL-6 (interleukin-6), IL-7 and TGF-β (transforming growth factors β) and can generate fat cells. Although their precise location in the bone marrow is not known it is thought that they may be located in the subendosteal area of the bone (reviewed by Kincade, 1990). The free, immature myeloid cells of the bone marrow include haemocytoblasts, erythroblasts, myeloblasts and megakaryocytes.

Circulation in the bone marrow is by means of sinusoids through which haemopoietic precursors pass into the bloodstream. The bone marrow is the chief source of multipotential stem cells for the repopulation of the spleen and thymus (McCulloch and Till, 1961; Micklem *et al.*, 1966) containing progenitors for various cell types including lymphoid cells, macrophages (Goodman, 1964) and dendritic cells (Steinman *et al.*, 1974). T cells derived from bone marrow precursors migrate to the thymus where they proliferate and differentiate into mature cells.

The thymus is very important in T cell development. As shown by the experiments of Miller (1961) neonatal thymectomy of mice produced immunologically-defective mature animals which had increased susceptibility to infections, a deficiency of cells in the lymph nodes and spleen, and an inability to reject unmatched skin grafts. In recent years, many studies have been published on the characterisation of cells in the thymus and the events necessary to produce immunocompetent T cells.

Immature T cells which enter the thymus (prothymocytes) undergo an ordered sequence of differentiation events producing mature T cells which migrate to peripheral lymphoid organs. These differentiation events include changes in cell surface antigens such as CD4, CD8 and the TcR (T cell receptor) with the formation of a thymocyte which is self-MHC (major histocompatibility complex) restricted but still self-tolerant. The mechanism of self-tolerance probably occurs through a clonal deletion process where self-reactive clones, during a critical stage in development, are eliminated from the population by certain thymic cells (reviewed by Sprent *et al.*, 1988). The prothmocyte which is phenotypically Thy-1^+/$CD4^-CD8^-$ has been poorly characterised but is thought to contain TcR chains in germline configuration (α and β antigen receptor genes before rearrangement) but does not express CD3 mRNA (Palacios *et al.*, 1987; Palacios and Pelkonen, 1988).

Thymocytes can be divided into three populations on the basis of CD4/CD8 expression. These are thymocytes that 1) express neither antigen ($CD4^-CD8^-$) from which is generated all CD4/CD8 phenotypes (Kingston *et al.*, 1985) and constitutes 3-5% of adult thymocytes (Ceredig *et al.*, 1983), 2) single positive populations with either the CD4 or CD8 antigen ($CD4^-CD8^+$, $CD4^+CD8^-$) which constitute 5-10% of adult thymocytes, and 3) a double positive population ($CD4^+CD8^+$) which constitutes 75-85% of adult thymocytes and is located in the cortical region of the thymus (Scollay *et al.*, 1984). Recent data (Kisielow *et al.*, 1988) has suggested that $CD4^+CD8^+$ thymocytes are the precursors of $CD4^-CD8^+$ and $CD4^+CD8^-$ thymocytes, however mechanisms involved in the regulation of expression of CD4 and CD8 are still unknown. Another interesting aspect of T cell development is the sequential assembly and expression of the antigen receptor complex (TcR and CD3) in the thymus (Snodgrass *et al.*, 1985a, 1985b).

The thymus is arranged into four distinct regions: 1) the outermost subcapsular cortex which is the major site of lymphopoiesis where large lymphocytes proliferate to produce thymocytes; 2) the inner cortex into which thymocytes migrate; 3) the medulla within the thymic parenchyma which contains mature cells; and 4) the perivascular connective tissue space surrounding larger medullary blood vessels. Besides lymphocytes, dendritic cells, macrophages and epithelial cells are also present in the thymus (Beller and Unanue, 1980; van Ewijk, 1988).

The thymus contains a highly organised system of blood vessels and a lymphatic drainage system which lies in the perivascular connective tissue space. Studies have shown that lymphocytes can leave this organ via the lymphatic route (Kotani *et al.*, 1966) or the venous route (Ernström *et al.*, 1965) It has been suggested that only a small percentage of lymphocytes migrate from the thymus, the rest dying within that organ (Matsuyama *et al.*, 1966; Metcalf and Wiadrowski, 1966).

Based on the brief outline presented above, it is clear that both the bone marrow and thymus represent highly organised and complex organs in which well defined patterns of cell migration and positioning occur. Virtually nothing is known about the molecular basis of this migration process.

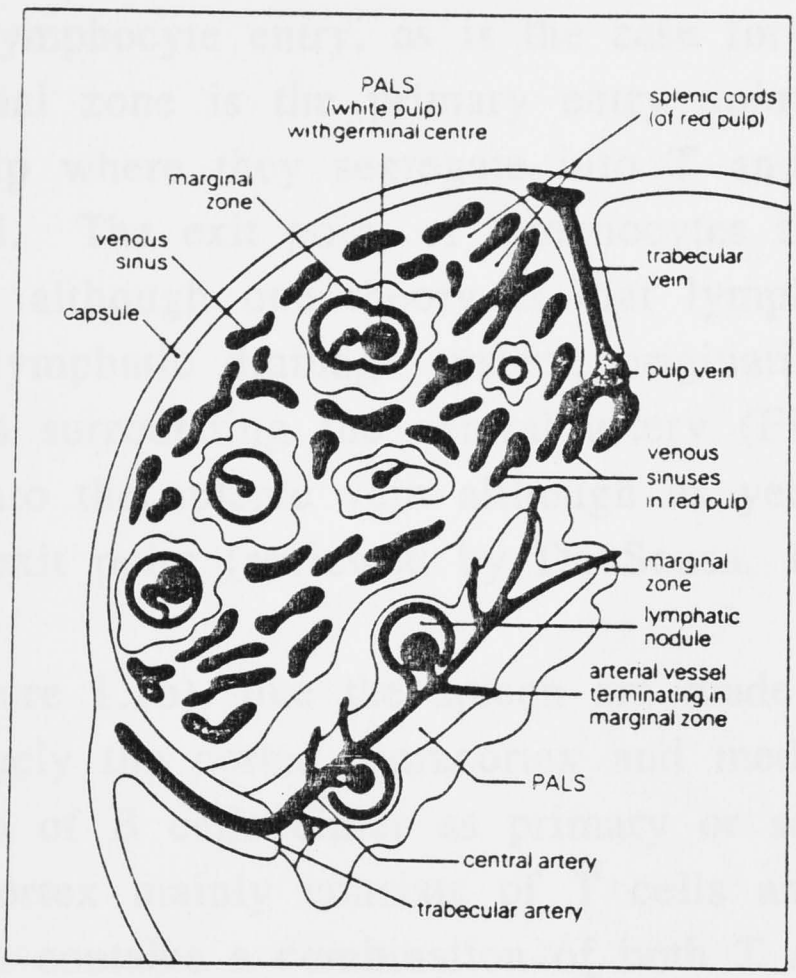
1.2.2 Secondary Lymphoid Organs

Secondary lymphoid organs include the spleen, lymph nodes and mucosal associated lymphoid tissue (MALT) which encompasses the Peyer's patches (PP) of the gut and the tonsils. The secondary lymphoid organs provide the appropriate microenvironment for the interaction of immunologically mature lymphocytes with foreign antigen and the subsequent development of an immune response.

The spleen (Figure 1.1a), although little studied, is a vital organ in lymphocyte recirculation and, presumably, presentation of foreign antigen to the immune system. More lymphocytes pass through the spleen than recirculate through all lymph nodes (Ford, 1968).

The spleen is made up of two main tissue types, the red and the white pulp. The red pulp is mainly concerned with destruction of effete erythrocytes and consists of sinuses lined by macrophages, lymphocytes and plasma cells. The white pulp or periarteriolar lymphoid sheath (PALS) is the main lymphocyte containing area and is arranged around a central arteriole. B and T cells are segregated in the PALS with the B cells predominantly found in germinal centres and the marginal zones, and the T cells being found around the central arteriole. The reticulum cells present in the PALS include dendritic cells in the B cell region, interdigitating cells in the T cell region and fibroblasts (reviewed by De Sousa, 1981). The marginal zones also contain macrophages which are distinct from the red pulp macrophages (Humphrey, 1980) and circulating granulocytes.

a)



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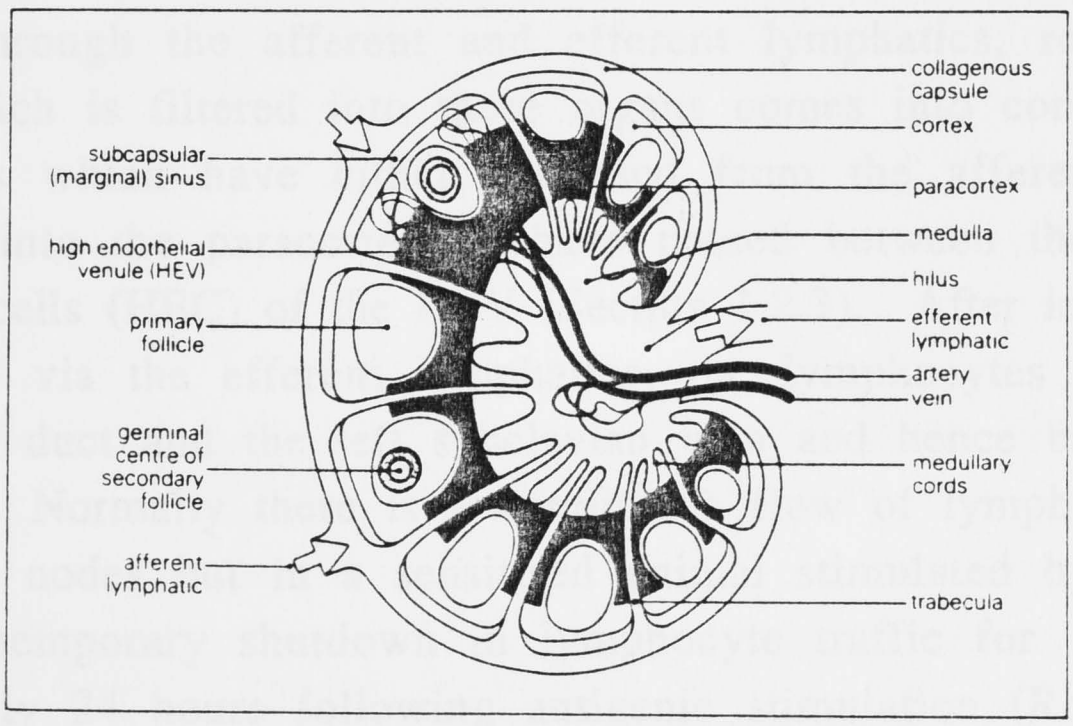


Figure 1.1 Structure of Lymphoid Tissue
a) Spleen and b) Lymph Node

Modified from Roitt *et al.*, 1989

The spleen is an unusual secondary lymphoid organ in that it does not contain HEV for lymphocyte entry, as is the case for lymph nodes. Instead the marginal zone is the primary entry point for lymphocytes into the white pulp where they segregate into T and B-dependent areas as mentioned. The exit point of lymphocytes from the spleen is basically unknown although one theory is that lymphocytes leave the white pulp by a lymphatic drainage system, originating in large lymphatic channels surrounding the central artery (Figure 1.2). These drain eventually into the splenic vein although as yet there is no evidence for this exit point (reviewed by De Sousa, 1981).

Lymph nodes (Figure 1.1b), like the spleen are made up of discrete cellular areas, namely the cortex, paracortex and medulla. The cortex contains aggregates of B cells either as primary or secondary follicles whereas the paracortex mainly consists of T cells and interdigitating cells. The medulla contains a combination of both T and B cells and phagocytes with plasma cells organised into medullary cords that run into the trabeculae and sinuses. Lymph enters and leaves the node at the hilus through the afferent and efferent lymphatics, respectively. Antigen which is filtered into these organs comes into contact with lymphocytes which have either traversed from the afferent lymphatics into the paracortex or have passed between the high endothelial cells (HEC) of the HEV (Section 1.2.3). After leaving the lymph node via the efferent lymphatics, the lymphocytes pass into the thoracic duct and the left subclavian vein and hence back into the circulation. Normally there is a continuous flow of lymphocytes through the nodes but in a sensitised animal stimulated by antigen, there is a temporary shutdown in lymphocyte traffic for approximately 24 hours following antigenic stimulation (Roitt *et al.*, 1985).

MALT consists of aggregates of lymphoid tissue which are not encapsulated as is the case with spleen and lymph nodes. They occur primarily in the gastrointestinal (PP), urogenital and respiratory tracts and are the main entry point for foreign organisms into the body.

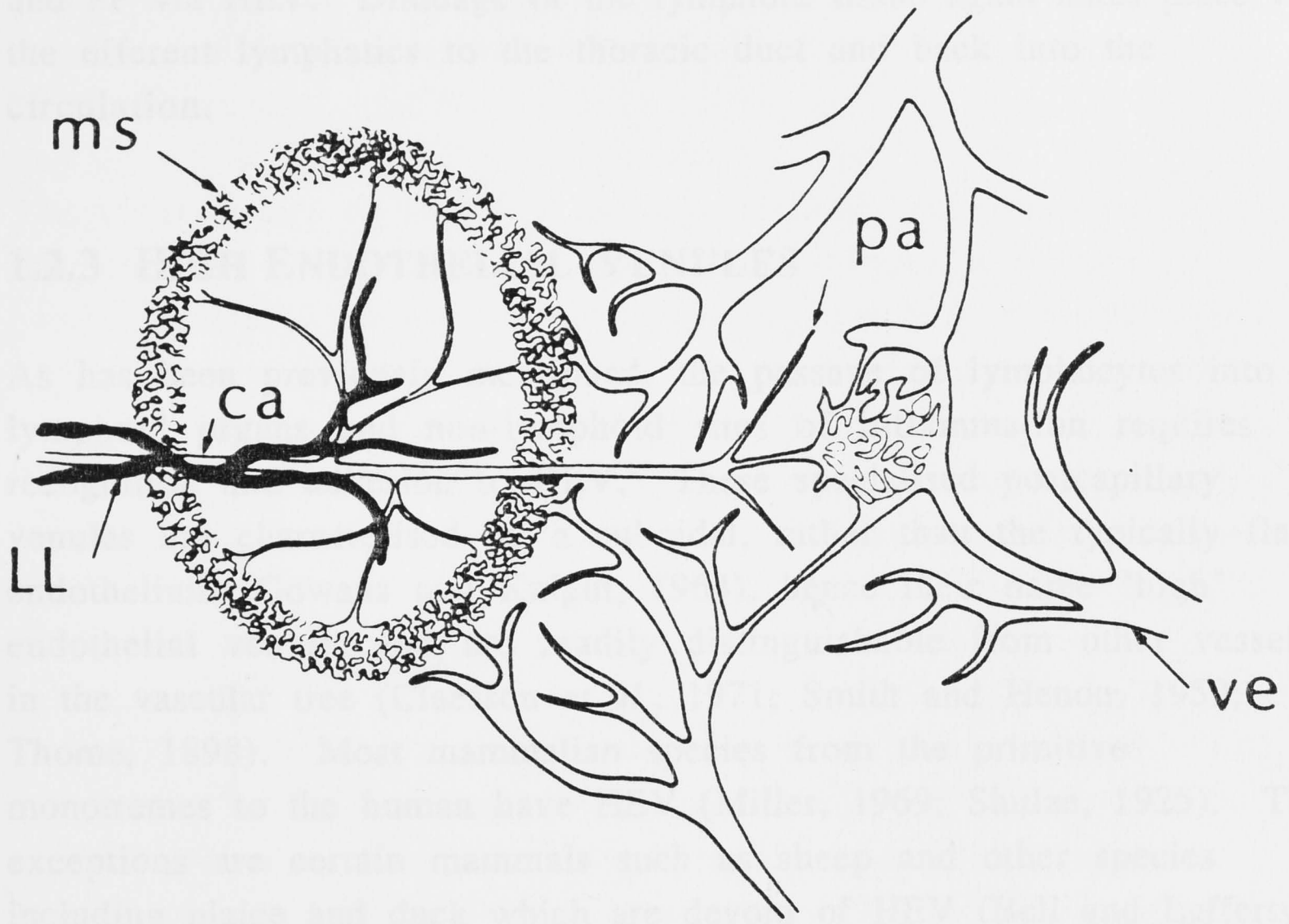


Figure 1.2 Diagrammatic Representation of the White Pulp Microcirculation

ca = central artery; ms = marginal sinus, close anastomosing irregular channels filling from capillaries branching off the ca; ve = veins of the red pulp; LL = lymphatic channels around the ca; pa = penicillae arteries originating at the ca (De Sousa, 1981).

Similar to other secondary lymphoid organs the PP can be divided into three regions. These are a nodular area occupied mainly by B cells, the interfollicular area which contains T cells and the dome area (De Sousa, 1981). Similar to lymph nodes, lymphocytes enter the tonsils and PP via HEV. Drainage of the lymphoid tissue again takes place via the efferent lymphatics to the thoracic duct and back into the circulation.

1.2.3 HIGH ENDOTHELIAL VENULES

As has been previously mentioned, the passage of lymphocytes into lymphoid organs and non-lymphoid sites of inflammation requires recognition and adhesion to HEV. These specialised postcapillary venules are characterised by a cuboidal, rather than the typically flat, endothelium (Gowans and Knight, 1964), hence their name "high" endothelial venule, and are readily distinguishable from other vessels in the vascular tree (Claesson *et al.*, 1971; Smith and Henon, 1959; Thome, 1898). Most mammalian species from the primitive monotremes to the human have HEV (Miller, 1969; Shulze, 1925). The exceptions are certain mammals such as sheep and other species including plaice and duck which are devoid of HEV (Bell and Lafferty, 1972; Ellis and De Sousa, 1974; Morris and Courtice, 1977; Schoefl, 1981), the functional significance of which is unknown.

HEV are found in all secondary lymphoid organs (with the exception of the spleen) including lymph nodes, PP, tonsils, adenoids, appendix, and small lymphoid aggregates associated with the mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts (Butcher and Weissman, 1984; Miller, 1969). However, they are absent from the primary lymphoid organs, although, they may be present in non-lymphoid tissues under certain circumstances such as during inflammation in mammals (Freemont, 1983; Freemont and Jones, 1983) and birds (Miller, 1969).

Histologically, HEC of the HEV are characterised by a number a interesting features. The HEC are cuboidal, 10-20 μm in diameter and linked by discontinuous junctions. This single layer of endothelium rests on a basement membrane which together with the associated sheath of reticular fibres appears to regulate the rate of lymphocyte migration into the lymphoid organ (Anderson and Anderson, 1975, 1976; Wenk *et al.*, 1974).

The ultrastructure of the HEC are also unusual, exhibiting the characteristics of metabolically active cells. Features include a prominent Golgi apparatus, numerous mitochondria, ribosomes and vesicles typical of active secretory cells, and a large nucleus with one or two nucleoli (Anderson and Anderson, 1975; Wenk *et al.*, 1974). Furthermore HEC can synthesise and secrete a sulfated glycolipid (Andrews *et al.*, 1982, 1983; Section 1.7.3), and have elevated levels of the enzymes, acid phosphatase, β-glucuronidase (Anderson *et al.*, 1976) and certain esterases (Smith and Henon, 1959).

Recently, monoclonal antibodies (mAbs) have been produced which are specific for HEV. These mAbs define the MECA series of HEV antigens which are thought to play a role in the binding of lymphocytes to HEV and have been termed "vascular addressins" (Streeter *et al.*, 1988a; Section 1.6.1).

1.3 RECIRCULATION OF LYMPHOCYTES THROUGH LYMPHOID ORGANS

Studies of lymphocyte recirculation revealed that the large numbers of cells leaving the thoracic duct and entering the circulation were sufficient to replenish the lymphocyte content of the blood several times daily (Sanders *et al.*, 1940; Yoffey, 1936). Since lymphocyte numbers in the blood remained constant, it was postulated that

lymphocytes were destroyed in the periphery (Hughes *et al.*, 1956; Yoffey, 1936) with associated, rapid lymphocyte proliferation elsewhere.

The concept that lymphocytes recirculate from blood to lymph was first suggested by Gowans (1957, 1959a, 1959b) who reinfused rats intravenously with their own lymphocytes taken from a previous thoracic duct drainage, and demonstrated that lymphocyte depletion could be prevented. Also, radiolabelled thoracic duct lymphocytes taken from donor animals and intravenously injected into recipient animals, reappeared in the lymph (Gowans, 1959a).

In 1964 Gowans and Knight finally demonstrated lymphocyte "homing" whereby lymphocytes left the blood and entered lymph nodes via postcapillary venules, returning to the bloodstream by way of the thoracic duct. The term "homing" was used to describe the migratory preference of lymphocytes for specific lymphoid organs. However, this term has some limitations for it implies previous familiarisation of the lymphocyte with the target lymphoid tissue. Other more generalised terms such as "migration" and "topographical memory" (Hamann and Thiele, 1989) may be more appropriate alternatives when the mechanism of lymphocyte recirculation is not inferred.

In studying lymphocyte recirculation the question arises as to why lymphocytes need to recirculate. Although this appears to be a purely philosophical question, the answer may lie in practicalities. For example, Kieran *et al.* (1989) have suggested that to maintain a generalised immunological surveillance of all body tissues is very costly. Discrete localisation of antigens in concentrated areas such as lymph nodes where the greatest number of antigen specific effector cells could see antigen, and specialised antigen presenting cells are localised, would minimise this cost to the body while affording maximum protection to the individual.

Thus lymphocyte recirculation maximises the probability of an interaction between an antigen trapped in a particular lymphoid organ interacting with the relatively small number of specific lymphocytes which can respond to it. The efficiency of this process is dependent on a continual and rapid recirculation of lymphocytes from the blood, into lymphoid tissue. For example in the rat 4×10^7 lymphocytes are returned to the blood via the thoracic duct per hour (Yednock and Rosen, 1989), which is sufficient to replace the blood content of lymphocytes 10-20 times per day (Ford, 1969; Gowans, 1959a). Also immunological tolerance to self antigens may be maintained during the process of lymphocyte recirculation, a topic on which little is known outside the primary lymphoid organs.

There are however, a number of normal physiological factors which can influence lymphocyte recirculation both specifically and non-specifically. For example, antigenic stimulation can affect lymphocyte homing in a variety of ways. Firstly, by increasing local blood flow which indirectly increases lymphocyte traffic through an area (Hay *et al.*, 1980). Secondly, it causes a transient delay in lymphocyte exit from a stimulated lymph node which thereby increases the number of lymphocytes in that area (Cahill *et al.*, 1976; Hall and Morris, 1965) and finally it selectively recruits antigen specific cells (Butcher, 1986). Another example is hormonal influences such as estrogen levels (Roux *et al.*, 1977; Weisz-Carrington *et al.*, 1978) which may influence lymphocyte migration.

1.4 SITE SPECIFIC MIGRATION OF LYMPHOCYTES

Although lymphocytes have the potential for universal migration they often display non-random, selective migration to certain lymphoid tissues (Butcher *et al.*, 1980; Chin and Hay, 1984; Stevens *et al.*, 1982) and sites within these tissues (De Sousa, 1981).

Since the classical study of Gowans and Knight (1964) where radiolabelled lymphoblasts selectively localised within mesenteric lymph node (MLN) and walls of the small intestine, a vast body of data has accumulated demonstrating selective lymphocyte homing and a number of extensive reviews have been published on the subject (Berg *et al.*, 1989; Butcher, 1986; Duijvestijn and Hamann, 1989; Hamann and Thiele, 1989; Jalkanen *et al.*, 1986b; Pals *et al.*, 1989; Woodruff and Clarke, 1987; Yednock and Rosen, 1989). The majority of these *in vivo* studies were performed using the adoptive transfer method with radiolabelled cells and it was not until 1976 that an *in vitro* method for measuring lymphocyte-HEV binding was developed by Stamper and Woodruff (summarised in Section 1.4.2).

1.4.1 *IN VIVO* STUDIES SHOWING LYMPHOCYTE BINDING SPECIFICITIES

The first *in vivo* line of evidence suggesting site specific migration of lymphocytes comes from the selective migration of lymphoblasts demonstrated in studies by Gowans and Knight (1964). In agreement with these early results was the work of many researchers (Chin and Hay, 1980; Griscelli *et al.*, 1969; Pierce and Cray, 1982; Rose *et al.*, 1976; Scollay *et al.*, 1976) and from this data developed the important concept of separate peripheral and mucosal immune systems where lymphocytes express receptors for either peripheral lymph nodes (PLN) or mucosal associated lymphoid organs.

For example, Scollay *et al.* (1976) showed that PLN or intestinal radiolabelled lymphoid cells from sheep showed preferences for PLN or gut lymphoid tissue, respectively and this was later confirmed by Chin and Hay (1980) Similar results were also obtained in mice using radiolabelled MLN or peripheral T blast cells (Rose *et al.*, 1976). Further specificity was shown when IgA-containing plasma cells were found to home to specific locations within the gut from which they were isolated (Pierce and Cray, 1982).

Additional studies have shown that lymphocytes may show specificity in migration for other tissues such as inflamed synovia, as indicated by the specific migration patterns of lymphocytes to inflammatory sites (Chin and Hay, 1980; Issekutz *et al.*, 1986; Rose *et al.*, 1976).

A number of reports have also indicated unique binding specificities for lung-associated lymphoid tissue. McDermott and Bienenstock (1979) have shown that lymphoblasts isolated from lung-associated lymphoid tissue selectively migrate back to that tissue. Furthermore, the early studies of Spencer *et al.* (1983) in the sheep were confirmed by Spencer and Hall (1984) who showed that rat, radiolabelled immunoblasts isolated from intrathoracic lymph nodes localised after intravenous injection preferentially to the lungs rather than the gut.

1.4.2 *IN VITRO.* STUDIES SHOWING LYMPHOCYTE BINDING SPECIFICITIES

The study of lymphocyte binding specificities was greatly aided by the development of an *in vitro* assay technique which could quantify the attachment of lymphocytes to HEV (Stamper and Woodruff, 1976). In this assay viable lymphocytes were layered onto cryostat sections of lymphoid tissue and their degree of binding determined by various methods (Butcher *et al.*, 1979a; Rosen *et al.*, 1985; Stamper and Woodruff, 1976). *In vitro* results were seen to closely parallel *in vivo* results (Butcher *et al.*, 1979b; Gallatin *et al.*, 1983; Woodruff and Clarke, 1987) demonstrating that specific HEV-lymphocyte interactions occurred *in vivo*. This assay has been widely used to compare different binding specificities between lymphocytes and lymphoid tissues and in the production of mAbs to lymphocyte receptors for HEV (Section 1.6).

As was the case with *in vivo* studies, specificity of lymphocyte/HEV interactions was demonstrated by the separate binding specificities of

lymphocytes for PLN and PP HEV. Butcher *et al.* (1980) showed *in vitro* (and *in vivo*) that about 1.4 times as many PP as lymph node lymphocytes bound to PP HEV and conversely, twice as many lymph node cells interacted with HEV in nonmesenteric lymph nodes. Even greater specificity was demonstrated with lymphoma cell lines that selectively bound to either PP or PLN HEV. MLN were found to be unique in that they bound lymphocytes with either PP or lymph node specificity. These findings led Butcher *et al.* (1980) to propose a generalised model for lymphocyte/HEV interactions (Section 1.5).

Another example of lymphocyte specificity is that demonstrated with the preferential binding of T cells to PLN HEV and B cells to PP HEV. Thus Stevens *et al.* (1982) showed that B cells from any source bound preferentially to PP HEV (twofold to threefold higher) and T cells of any type to PLN HEV (1.5 fold higher). Results of *in vivo* studies of PLN and PP selective homing were found to resemble closely those of *in vitro* HEV-binding preferences (Kraal *et al.*, 1983; Stevens *et al.*, 1982). Furthermore *in situ*, PLN contain a greater proportion of T cells than B cells with the opposite being true for PP (Yednock and Rosen, 1989). These results indicated that specific receptors for PLN and PP were present on each cell type with one receptor being present to a higher degree than the other (Yednock and Rosen, 1989). This led Stevens *et al.* (1982) to suggest that lymphocyte/HEV binding specificity would regulate lymphocyte localisation within a tissue and hence the type of immune response generated within that organ. Also differences were seen between the ability of the $CD4^+$ and $CD8^+$ T cell subsets to bind to PLN HEV (Kraal *et al.*, 1983).

Although the specificity of homing of T and B cells was seen to differ, it has been suggested that this may reflect the presence of memory cells in the population and not unprimed cells which may exhibit several homing receptors that are then selected for by antigen stimulation (Butcher, 1986; Yednock and Rosen, 1989). This theory is supported by a number of lines of evidence. Firstly, Hall *et al.* (1977) showed that lymphoblasts isolated from adults homed specifically

when injected into foetal animals, whereas foetal T cells were not seen to home (Cahill *et al.*, 1979). Secondly, lymphocytes obtained from lymph node and spleen (recirculating cells) specifically adhered to frozen sections of HEV (Stamper and Woodruff, 1976) whereas lymphocytes obtained from bone marrow and thymus (non-recirculating cells, immature cells) could not. This apparent specificity difference between T and B cells adhering to HEV is not the result of differing affinities for HEV as both affinity and rate of binding are of similar magnitude (Kuttner and Woodruff, 1979) nor is this specificity absolute, for a small subpopulation of thymocytes has been shown to have homing abilities *in vivo* (Zatz and Lance, 1970) and similarly *in vitro* (Butcher *et al.*, 1979a).

Generally therefore, most immature lymphocytes from bone marrow and thymus do not have homing receptors, while mature lymphocytes, not yet primed from spleen or lymph node do, and can bind to both mucosal and peripheral lymphoid tissue. Antigen-specific memory cells however, show very selective migratory behaviour moving to those tissues originally involved in antigen exposure and activation. For example, isolated intraepithelial lymphocytes of the gut home exclusively to gut and gut associated lymphoid tissue (GALT) such as PP (Schmitz *et al.*, 1988).

As was discussed previously, lymphocytes have been shown to have specificity for inflamed synovia *in vivo* (Chin and Hay, 1980; Issekutz *et al.*, 1986; Rose *et al.*, 1976). This has been also shown *in vitro* by Jalkanen *et al.* (1986c) who demonstrated that lymphocytes bound specifically to inflamed synovia HEV, an interaction which was not inhibited by antibodies against the receptors on lymphocytes specific for either PLN HEV or GALT HEV. Furthermore, lymphoblastoid cell lines which bound to either PLN or GALT were unable to bind to synovial HEV, which suggested a separate HEV receptor in inflammatory sites (Jalkanen *et al.*, 1986c).

Geoffroy *et al.* (in preparation, quoted by Yednock and Rosen, 1989) confirmed that lung specificity was separate from that of PLN and PP, in a series of experiments where treatments that selectively inhibited PLN or PP HEV attachment were applied to lung-associated lymphoid tissue. No inhibition of lymphocyte binding to lung HEV was seen, confirming that this specificity is unique.

1.5 MOLECULAR MODELS OF LYMPHOCYTE HOMING

As has been previously stated lymphocytes migrate and localise selectively to particular lymphoid organs, leaving the blood by means of specialised HEV. The specificity of migration has long been thought to be due to specific receptors on the lymphocyte surface which bind to complementary structures on the HEV surface.

Based on these concepts a model for lymphocyte specificity was developed initially by Butcher *et al.* (1980), and then modified by Stevens *et al.* (1982). The latter proposed that at least three complementary lymphocyte-HEV receptor sets exist namely, a set involved in PLN entry, a set associated with PP and appendix entry, and finally a set involved in lymphocyte entry into inflamed synovium. It would appear that a lung-associated specificity also exists (Geoffroy *et al.*, in preparation; McDermott and Bienenstock, 1979; Spencer and Hall, 1984) and other possible tissue specificities are still being investigated. Also some lymphoid organs such as MLN, exhibit a mixture of entry specificities which, in the case of MLN is consistent with receptors for both PLN and PP HEV entry.

A simple molecular model depicting this mechanism of lymphocyte homing with different receptor specificities is proposed in Figure 1.3 (modified from Yednock and Rosen, 1989). However, this model does have some drawbacks in that it does not take into account the role of

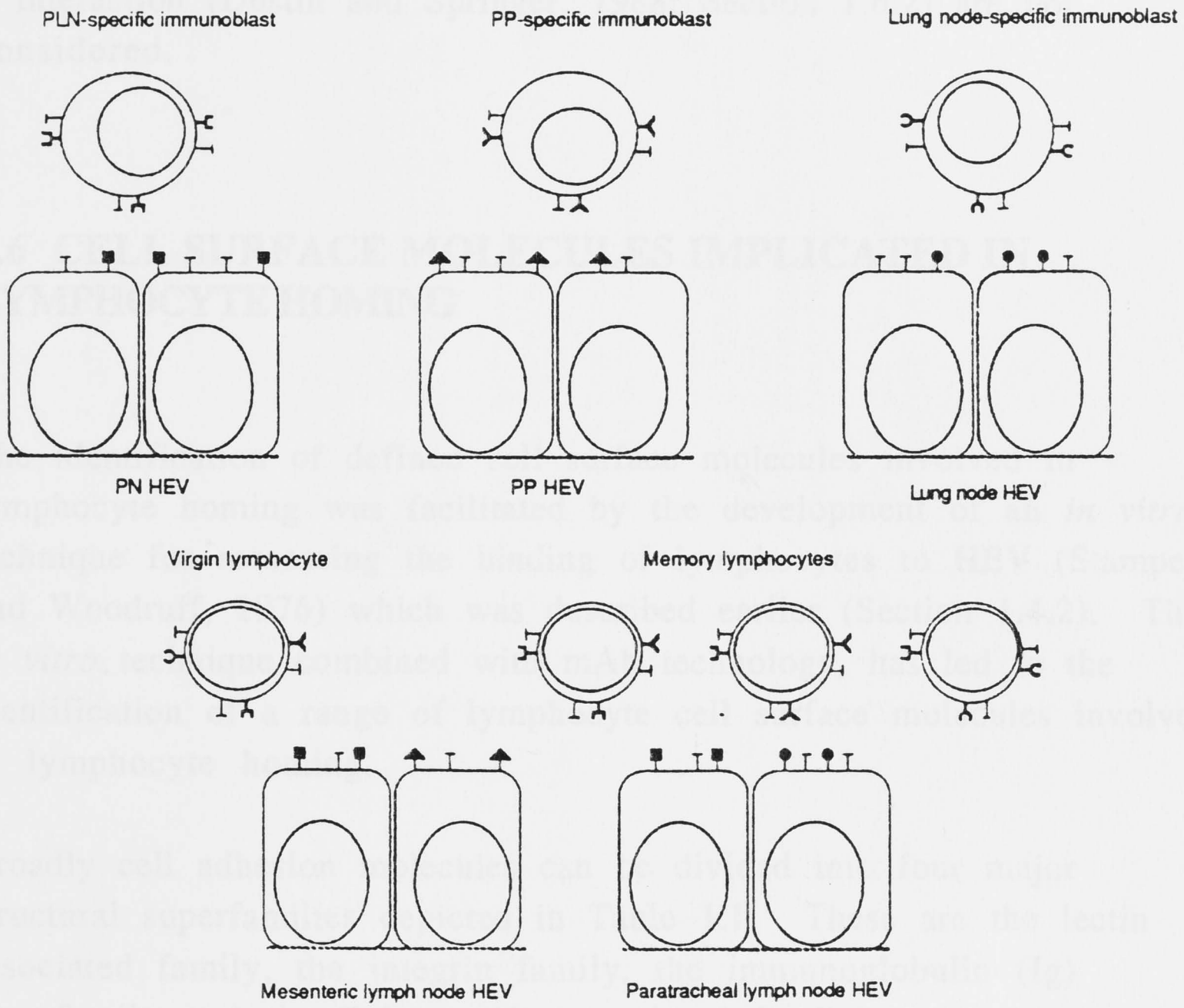


Figure 1.3 Model for Organ-Specific Lymphocyte Attachment to HEV

(Yednock and Rosen, 1989)

soluble enhancers of *in vitro* lymphocyte binding found in lymph, such as adherence enhancing factor (Carey *et al.*, 1981) and high endothelial binding factors for PP (HEBFPP, 1B.2) and PLN (HEBFLN, A.11 antigen)(Chin *et al.*, 1986; Rasmussen *et al.*, 1985; Woodruff and Clarke, 1987). Furthermore, additional receptor-ligand interactions which may augment organ-specific binding, such as the LFA-1/ICAM-1 interaction (Dustin and Springer, 1988; Section 1.6.2) are not considered.

1.6 CELL SURFACE MOLECULES IMPLICATED IN LYMPHOCYTE HOMING

The identification of defined cell surface molecules involved in lymphocyte homing was facilitated by the development of an *in vitro* technique for measuring the binding of lymphocytes to HEV (Stamper and Woodruff, 1976) which was described earlier (Section 1.4.2). This *in vitro* technique combined with mAb technology, has led to the identification of a range of lymphocyte cell surface molecules involved in lymphocyte homing.

Broadly cell adhesion molecules can be divided into four major structural superfamilies depicted in Table 1.1. These are the lectin associated family, the integrin family, the immunoglobulin (Ig) superfamily and the link protein associated family.

The **lectin associated family** is characterised by a lectin-like domain at their amino-terminal end followed by an epidermal growth factor (EGF) -like domain and short repeating protein units. Members of this family include MEL-14 (Section 1.6.1), LAM1 (leukocyte adhesion molecule 1), GMP-140 (human granule-membrane protein) expressed by activated platelets and endothelial cells (Johnston *et al.*, 1989) and ELAM-1 (endothelial leukocyte adhesion molecule 1).

Table 1.1 Cell Adhesion Families

Family	Molecule involved in adhesion	Distribution
Lectin associated	MEL-14 antigen (mLHR)	Murine lymphocytes
	LAMI	Human leukocytes
	ELAM-1	Human endothelium of immune/inflammatory lesions
	GMP-140 (CD62)	Human activated platelets and endothelium
	Ca-dependent lectins	Human and rodent lymphocytes and malignant lymphoid lines
Integrins	LFA-1 (CD11a/18, Ly15)	Human and murine leukocytes
	LPAM-1 (VLA-4)	Human and murine lymphocytes and malignant lines
	CR3 (CD11b/18, Mac1, Mo1, OKM1, Leu15)	Human and murine leukocytes
	p150,95 (CD11c/18)	Human leukocytes
	GPIIb (GPIIIa)	Platelet plasma membrane
Immunoglobulins	ICAM-1 (CD54, 7F7-antigen)	Human endothelium, epithelium and fibroblasts
	ICAM-2	Human endothelium and lymphoblastoid cell lines
	CD2 (T11, LFA-2, SRBC rosette receptor)	Human T cells
	CD4 (T4, Leu3, L3T4)	Human and murine T cells restricted to class II MHC
	CD8 (T8, Leu2, Ly2)	Human and murine T cells restricted to class I MHC
Link protein associated	CD44 (HCAM, Hermes antigens, Pgp-1, gp^{90}Hermes, CR/ECMRIII, In(Lu)-related p80, Hutch1, Ly24)	Human and non-human primates and murine haematopoietic, epithelial and mesenchymal cells and related lines.

LAM1, which is expressed on human lymphocytes, may be involved in lymphocyte extravasation into lymphoid organs or sites of acute inflammation (Tedder *et al.*, 1990). The LAM1 amino acid sequence is highly homologous with that of the MEL-14 antigen suggesting that they may share a common function (Tedder *et al.*, 1989). Furthermore, the LAM1 molecule reacts with the TQ1 and Leu-8 mAbs.

ELAM-1 which has recently been cloned (Bevilacqua *et al.*, 1989) is expressed by activated human endothelial cells and mediates the adhesion of neutrophils to the lining of blood vessels stimulated by inflammatory lymphokines.

The **integrin family** of cell surface receptors is characterised by two noncovalently associated transmembrane polypeptides, termed subunits α and β. It contains several adhesion molecules (chicken integrin complex, fibronectin (FN) receptor, vitronectin (VN) receptor and glycoprotein IIb/IIIa) involved in recognition of extracellular matrix (ECM) proteins such as FN, VN, fibrinogen (FB) and von Willebrand factor (VWF). Many of these are identified by their ability to bind these ligands at sites encompassing the tripeptide sequence arginine-glycine-aspartic acid (RGD; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Based on structural and sequence homology a number of leukocyte cell surface antigens are also included in this family.

In terms of the immune system two sets of integrin receptors are of particular interest. The first is restricted to leukocytes and consists of LFA-1 (lymphocyte function-associated antigen-1), Mac-1, and p150/95 (reviewed by Anderson and Springer, 1987). LFA-1, which will be discussed further in Section 1.6.2, has an accessory function in the adhesion of lymphocytes to HEV (Harder and Heinz-Günther, 1988). Mac-1 and p150/95 mediate monocyte and granulocyte adhesion to endothelial cells and other substrata.

The second is the VLA (very late antigens; VLA-1, -2, -3, -4 and -5) series of antigens which are expressed on a variety of human cell types such as lymphocytes, and include receptors for ECM proteins such as FN, laminin and collagen (Hemler, 1988; Takada *et al.*, 1987, 1988). Each VLA antigen is composed of unique α-subunits in association with a common 13,000 molecular weight (MW) β subunit. LPAM-1 (lymphocyte PP HEV adhesion molecule-1) which showed similarities to VLA-4 (Holzmann *et al.*, 1989), is involved in the adhesion of murine lymphocytes to PP HEV.

The **Ig superfamily** contains a very broad range of functionally diverse molecules including Ig, TcR, MHC class I and II, CD1, CD2, CD3, CD4, CD8, LFA-3, Thy-1, NCAM (neural cell adhesion molecule), ICAM-1 (intercellular adhesion molecule-1) and ICAM-2 (Anderson *et al.*, 1988; Calabi, 1987). These are grouped together in the superfamily on the basis of structural homology between disulfide-bonded protein domains rather than DNA sequence. Ig domains show related but distinct folding patterns in their variable (V) and constant (C) regions. In both V and C domains two β-sheets stabilised by the disulfide bond, form the Ig fold, however the V-domain fold has an extra loop in the middle forming the second hypervariable region in antibodies (Williams, 1987). Sequence homology can be common to both V and C domains or specific to either the V or C domain types.

The Igs which are typical examples of this family consist of two light and four heavy chain domains containing V and C regions and, like many members of the superfamily, they may possess multiple domains (Calabi, 1987).

Members of the Ig superfamily such as ICAM-1 and ICAM-2 have been implicated as secondary adhesion molecules involved in lymphocyte migration (Section 1.6.2). Also it has been suggested that members of the Ig superfamily are involved with sulfated polysaccharides (SPS). For example, CD4 binds to dextran sulfate and

heparin (Lederman *et al.*, 1989) and Class II MHC is a chondroitin sulfate proteoglycan (Sant *et al.*, 1985).

Finally, there is the **link protein associated family** of which the only known cell adhesion molecule is CD44 (Section 1.6.1). Cloning data (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989) has shown CD44 to be a member of this family where the amino-terminal, extracellular domain sequence showed structural homology with cartilage link proteins and a related segment of proteoglycan core protein.

Relevant members of these superfamilies which are involved in lymphocyte homing will be discussed in more detail below (see also Table 1.2).

1.6.1 Specific Adhesion Molecules

MEL-14 As previously mentioned, lymphoma cell lines which exhibit specific adherence for HEV (Table 1.2) have been used to study lymphocyte homing at the molecular level. The identification of the MEL-14 antigen is one of the first examples of this approach where a rat mAb was raised against the mouse lymphocyte adherence receptor for PLN HEV.

In this case the PLN specific B cell lymphoma cell line, 38C-13 (Bergman and Haimovich, 1977; Haran-Ghera and Peled, 1973), was used to immunise Fischer rats and the immune rat spleen cells were then fused with the SP20 mouse myeloma to generate hybridomas (Gallatin *et al.*, 1983). Supernatants taken from these hybridomas were tested by immunofluorescence for their reactivity with the surface of 38C-13 cells and normal lymphocytes, and for the absence of reactivity with two non-binding lymphoma cell lines (Raw112 and EL-4). Antibodies from one hybridoma termed MEL-14, were found to react with lymphoma cell lines that were capable of binding to PLN HEV (Table 1.2).

Table 1.2 Lymphocyte Adhesion Molecules

Molecule	Endothelial adhesion site	Size (kDa)	Ligand
MEL-14	PLN HEV and MLN HEV	80-90, 180	M6P
Ca-dependent lectins	PLN HEV and MLN HEV	90?,180	M6P, F1P, PPME, fucoidin, sialyloligosaccharide on HEV
A.11 antigen ($HEBF_{LN}$)	PLN HEV and MLN HEV	40, 60, 135	Unknown
LPAM-1 (VLA-4)	PP HEV and MLN HEV	160, 130	Extracellular matrix ?, MECA-367 antigen ?
1B.2 antigen (HEBFPP)	PP HEV and MLN HEV	80	Unknown
CD44 (HCAM, Hermes antigens, Pgp-1, gp^{90}Hermes, CR/ECMR111, In(Lu)-related p80, Hutch1, Ly24)	Multiple tissue endothelia	80-95, 160, 180-200	Extracellular matrix ?
LFA-1 (CD11a/18, Ly15)	Multiple tissue endothelia	95,180	ICAM-1, other HUVE ligand(s)
ICAM-1	Multiple tissue endothelia	90	LFA-1
ICAM-2	Multiple tissue endothelia	46	LFA-1

M6P = mannose-6-phosphate, F1P = fructose-1-phosphate, PPME = phosphomannan ester, HUVE = human umbilical vein endothelium
Table modified from Stoolman (1989)

Furthermore when 38C-13 cells that were previously incubated with the MEL-14 mAb were tested for binding to PLN HEV it was found that binding was almost completely inhibited. MLN lymphocytes, which bind to both PLN and PP HEV, were then pretreated with MEL-14 and incubated on sections of PLN and PP HEV. It was found that binding of lymphocytes to PLN but not PP HEV was blocked by MEL-14 pretreatment. This is in agreement with the model proposed by Stevens *et al.* (1982) where MLN HEV carries separate lymphocyte binding receptors for both PLN and PP HEV (Figure 1.3). Similar results were obtained *in vivo,* which supported the view that the MEL-14 antigen is necessary for homing of lymphocytes to the PLN in mouse.

These results were further confirmed by experiments where intraperitoneal injection of MEL-14 greatly reduced (by 10-20 fold) the size of enlarged PLN observed in MLR-lpr/lpr mice, presumably by blocking the homing of cells to the PLN (Mountz *et al.*, 1988).

Characterisation of the MEL-14 antigen showed that it is a glycoprotein varying in MW from 80,000 on lymphocytes to 92,000 on the lymphoma cell line $gp90^{MEL\text{-}14}$ (Gallatin *et al.*, 1983) and 100,000 on neutrophils (Lewinsohn *et al.*, 1987). Its pI range is from 4.0-4.5 and it contains intrachain disulfide bonds (Siegelman *et al.*, 1986). The molecule is heavily glycosylated as shown by endoglycosidase F digestion (Siegelman *et al.*, 1986). An important property of the MEL-14 antigen is that it is a lectin (Stoolman *et al.*, 1984) recognising carbohydrate moieties on the surface of PLN HEV (Yednock *et al.*, 1987b). This aspect will be more fully discussed in Section 1.7.3.

The MEL-14 antigen has recently been cloned and sequenced (Lasky *et al.*, 1989; Siegelman *et al.*, 1989) and was found to be an integral membrane protein with the amino-terminal amino acid sequence having sequence homology with the carbohydrate domain of some Ca^{2+} dependent lectins (Drickamer, 1988). This is followed by an EGF-

like domain of 33 amino acids and two identical 62 amino acid repeats (Figure 1.4).

The MEL-14 antigen has a high amino acid sequence homology with that of another member of the lectin associated family, the LAM1 molecule (Tedder *et al.*, 1989, 1990).

MEL-14 antigen expression changes during lymphocyte development. Reichert *et al.* (1984, 1986) found that only 3-6 % of thymocytes, mostly mature, have high MEL-14 levels and similarly, only mature B and T cells in other lymphoid organs and the blood express high levels of MEL-14 antigen. Antigen-activated lymphocytes, which lose their lymph node homing ability, were found to have much lower surface expression of MEL-14 (Dailey *et al.*, 1985). Thus the MEL-14 antigen is associated with mature, recirculating, unstimulated lymphocytes.

Screening of bacteriophage λgt11 cDNA libraries (constructed using 38C-13 cell line mRNA; St. John *et al.*, 1986) for MEL-14 antigen expression, resulted in the isolation of cDNA clones encoding the ubiquitin sequence. This result suggested that ubiquitin may be part of the MEL-14 antigen. Ubiquitin is an evolutionarily highly conserved (Gavilanes *et al.*, 1982) polypeptide of MW 8451 first isolated from bovine thymus (Goldstein *et al.*, 1975). Its suggested functions have included control of gene transcription and organisation of chromatin structure, regulation of intracellular protein degradation, and control of mitosis (Siegelman *et al.*, 1986). Partial amino acid sequencing of the MEL-14 antigen revealed the presence of two amino termini, one of which corresponded exactly to the amino terminus of ubiquitin (Siegelman *et al.*, 1986). This was the first demonstration of a ubiquitinated cell surface protein. The functional significance of this observation is unclear. However, it has been suggested that ubiquitination may signal rapid degradation of the MEL-14 antigen, possibly when the molecule is internalised by the cell following ligand binding.

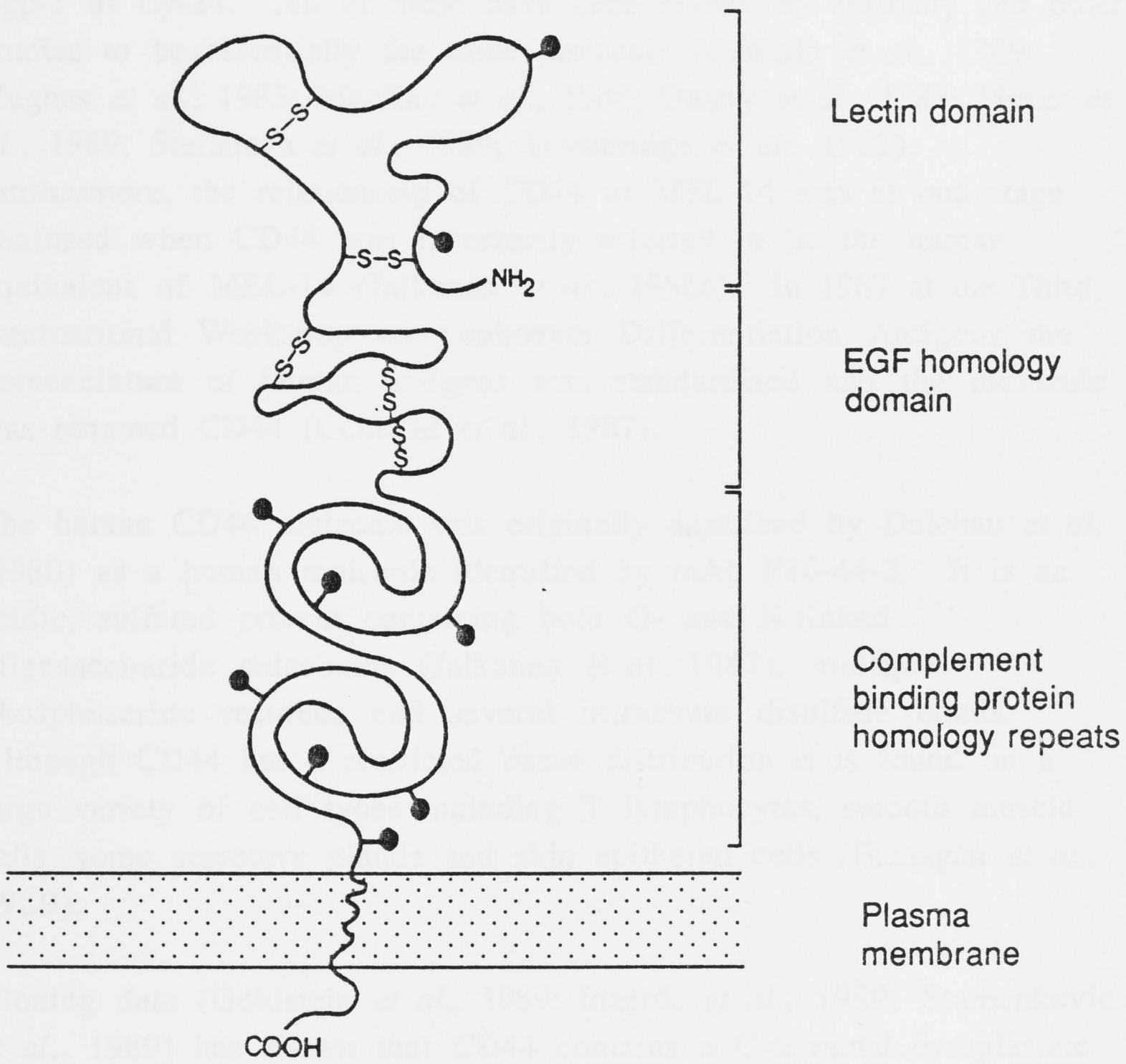


Figure 1.4 MEL-14 Antigen

Putative N-glycosylation site ⊸ ;
Modified from Coombe and Rider (1989)

CD44 CD44 has been previously known under a variety of alternative names which have caused confusion e.g. in the human, In(Lu)-related p80, ECMRIII (extracellular matrix receptor III), Hermes antigens, HCAM and gp90 Hermes; in the macque Hutch1; and in the mouse, Pgp-1 or Ly-24. All of these have been shown by antibody and other studies to be essentially the same molecule (Gallatin *et al.*, 1989; Hughes *et al.*, 1983; MacKay *et al.*, 1988; Omary *et al.*, 1988; Picker *et al.*, 1989; Stefanova *et al.*, 1989; Trowbridge *et al.*, 1982). Furthermore, the relationship of CD44 to MEL-14 was at one stage confused when CD44 was incorrectly inferred to be the human equivalent of MEL-14 (Jalkanen *et al.*, 1986c). In 1987 at the Third International Workshop on Leukocyte Differentiation Antigens the nomenclature of human antigens was standardised and the molecule was renamed CD44 (Cobbold *et al.*, 1987).

The human CD44 molecule was originally described by Dalchau *et al.* (1980) as a human molecule identified by mAb F10-44-2. It is an acidic, sulfated protein containing both O- and N-linked oligosaccharide sidechains (Jalkanen *et al.*, 1987), multiple phosphoserine residues and several intrachain disulfide bonds. Although CD44 has a restricted tissue distribution it is found on a large variety of cell types including T lymphocytes, smooth muscle cells, some secretory glands and skin epithelial cells (Flanagan *et al.*, 1989).

Cloning data (Goldstein *et al.*, 1989; Idzerda *et al.*, 1989; Stamenkovic *et al.*, 1989) has shown that CD44 contains a C-terminal cytoplasmic tail, a hydrophobic transmembrane domain of 23 amino acids and a N-terminal extracellular region of 248 amino acids shown in Figure 1.5 (Coombe and Rider, 1989). The 150 amino acid proximal extracellular domain contains two putative N-glycosylation sites and five putative O-glycosylation sites and is the region thought to contain four sites for chondroitin sulfate linkage through serine-glycine motifs. The 90 amino acid distal region of CD44 contains the majority of potential N-glycosylation sites and cysteines and exhibits homology to the

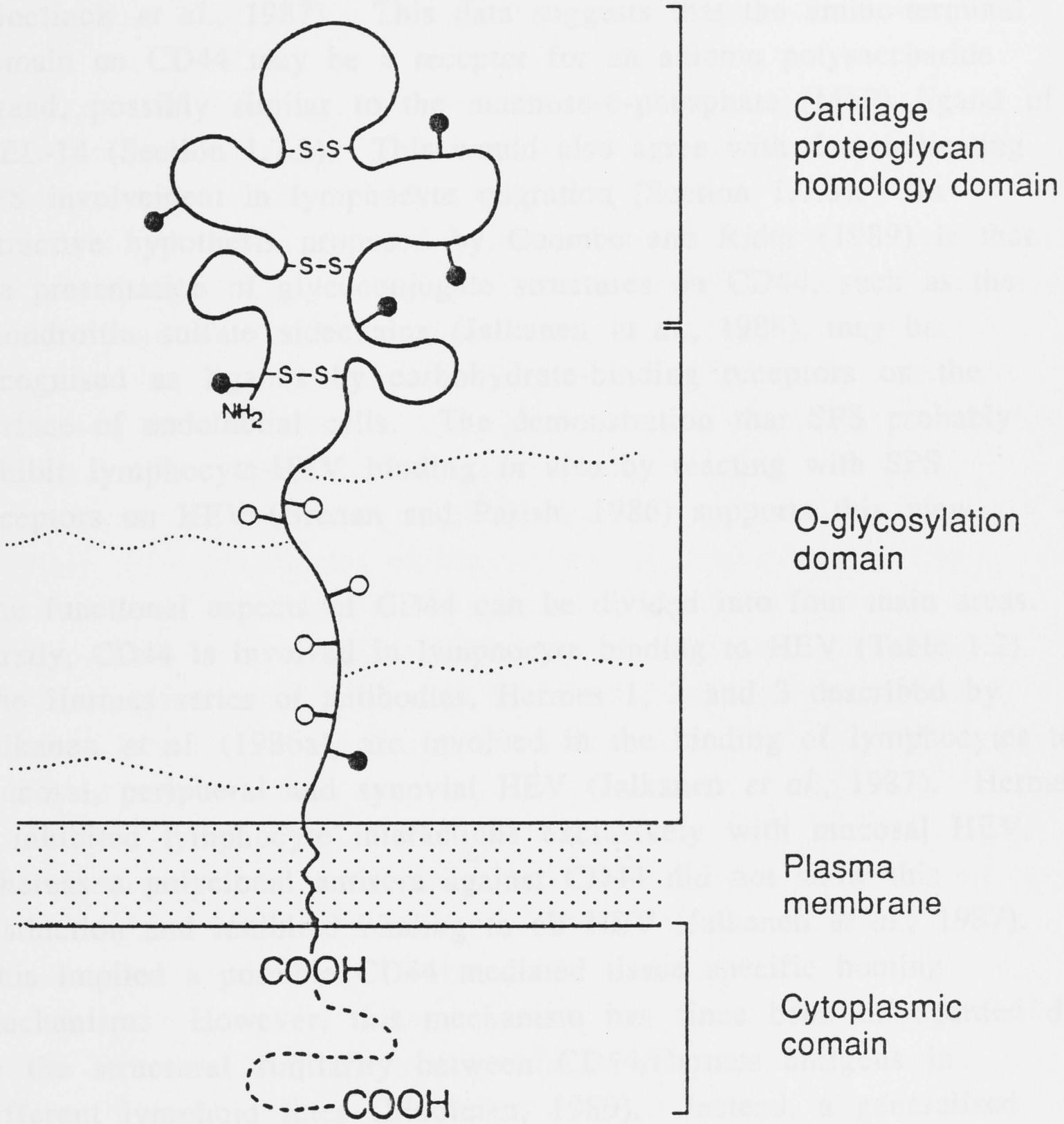


Figure 1.5 CD44

Putative N-glycosylation site —● ; putative O-glycosylation site —○ ; chondroitin sulfate chain in proteoglycan variant ..... ;
Modified from Coombe and Rider (1989)

cartilage link protein and proteoglycan core proteins (as mentioned previously) which are involved in protein-hyaluronic acid interactions (Goetinck *et al.*, 1987). This data suggests that the amino-terminal domain on CD44 may be a receptor for an anionic polysaccharide ligand, possibly similar to the mannose-6-phosphate (M6P) ligand of MEL-14 (Section 1.7.3). This would also agree with data indicating SPS involvement in lymphocyte migration (Section 1.7.3). An attractive hypothesis proposed by Coombe and Rider (1989) is that the presentation of glycoconjugate structures on CD44, such as the chondroitin sulfate sidechains (Jalkanen *et al.*, 1988), may be recognised as ligands by carbohydrate-binding receptors on the surface of endothelial cells. The demonstration that SPS probably inhibit lymphocyte-HEV binding *in vivo* by reacting with SPS receptors on HEV (Brenan and Parish, 1986) supports this view.

The functional aspects of CD44 can be divided into four main areas. Firstly, CD44 is involved in lymphocyte binding to HEV (Table 1.2). The Hermes series of antibodies, Hermes 1, 2 and 3 described by Jalkanen *et al.* (1986a), are involved in the binding of lymphocytes to mucosal, peripheral and synovial HEV (Jalkanen *et al.*, 1987). Hermes 3 inhibited lymphocyte interactions exclusively with mucosal HEV, whereas a polyclonal antisera against CD44 did not show this distinction and inhibited binding to all HEV (Jalkanen *et al.*, 1987). This implied a possible CD44 mediated tissue specific homing mechanism. However, this mechanism has since been disregarded due to the structural similarity between CD44/Hermes antigens in different lymphoid lines (Stoolman, 1989). Instead, a generalised hypothesis has been proposed by Holzmann *et al.* (1989) that the organ specific component of the lymphocyte/HEV interaction is mediated by multiple adhesion molecules, where several adhesion molecules may have to operate in conjunction to allow lymphocyte recognition and entry into an organ.

Secondly, CD44 may act as a link between extracellular matrix components and the cytoskeleton of cells. ECMRIII was described by

Carter (1982), as a 90 kDa surface molecule of human fibroblasts. It was proposed to link the fibroblast cytoskeleton with the ECM through binding both collagen type I and VI and FN (Carter & Wayner, 1988).

Thirdly, CD44 may regulate cell adhesion systems *in vivo* via the CD2/LFA-3 interaction. LFA-3 on sheep and human erythrocytes interacts with CD2 on T cells resulting in E-rosette formation. Antibodies to CD44 inhibit this interaction by binding to erythrocytes and modifying the ability of LFA-3 to interact with CD2 (Shimizu *et al.*, 1989). Such observations suggested that CD44 may be in close proximity to LFA-3 on the erythrocyte surface or binding of antibodies to CD44 may in some way induce a conformational change in LFA-3 (Haynes *et al.*, 1989).

Fourthly, CD44 molecules on the surface of T lymphocytes may regulate the CD2/LFA-3 interaction and thereby be involved in regulation of T cell activation. Huet *et al.* (1989) and Shimizu *et al.* (1989) both observed that mAb to CD44 dramatically augmented T cell proliferation and IL-2 synthesis induced by CD3 and CD2 receptor mediated activation.

Vascular Addressins Vascular addressins are the complementary receptor glycoproteins on the surface of HEV that provide a target structure for lymphocyte receptors (Streeter *et al.*, 1988a). Tissue specific mAbs to these addressins have been produced. For example, MECA-367 and MECA-89 are specific for vascular addressins on murine PP HEV whereas MECA-79 is specific for a vascular addressin on murine PLN HEV (Streeter *et al.*, 1988a, b). MECA-367 immunoprecipitates a 58-66 kDa protein, that contains disulfide bonds, possesses a heterogeneous pI of 6.7, 6.8 and 6.9 and is N- and O-glycanase resistant. In contrast, MECA-79 recognises a 92 kDa molecule, unchanged by reduction, with a pI of 6.2 and which is N-glycanase sensitive (Berg *et al.*, 1989). Currently the nature of the cell surface receptors on lymphocytes for these vascular addressins has

not been established, although strong candidates are MEL-14 (human LAM1) and CD44.

1.6.2 SECONDARY ADHESION MOLECULES

LFA-1 LFA-1 (CD11a/CD18) is a member of the integrin family (see Table 1.1), found exclusively on leukocytes (Hynes, 1987). It has an α and β chain of MW 180,000 and 95,000 respectively, arranged in a heterodimer complex. Studies by Hamann *et al.* (1988) have revealed that antibodies to LFA-1 inhibit lymphocyte attachment to HEV *in vitro* and *in vivo* where the migration of normal lymphocytes into lymph nodes and PP was decreased by 40-60%. Localisation to the lung was also impaired. Cells that contained high levels of the MEL-14 antigen were less susceptible to inhibition by anti-LFA-1 than those with low levels, which supports the function of LFA-1 as an accessory molecule supporting weak interactions between cells (Harder and Heinz-Günther, 1988). There is also some evidence that LFA-1 expression is required for metastasis to occur in certain lymphoma cell lines (Roossien *et al.*, 1989). This secondary function of LFA-1 is supported by its role in stabilising cell adhesion in many other forms of leukocyte adhesion (Springer *et al.*, 1987).

ICAM-1/ICAM-2 ICAM-1 (CD54) was identified as a B cell activation marker (Clark *et al.*, 1986) and a putative ligand for LFA-1 (Table 1.2) expressed on B-lymphoblastoid cells (Rothlein *et al.*, 1986), an observation subsequently confirmed by Simmons *et al.* (1988). It is a single chain glycoprotein of MW 90,000, constitutively expressed on some tissues and induced on others in inflammation (Boyd *et al.*, 1988; Dustin *et al.*, 1988; Dustin and Springer, 1988; Marlin and Springer, 1987). Another ligand to LFA-1 which has been cloned recently by Staunton *et al.* (1989) is ICAM-2. Both ICAM-1 and ICAM-2 are members of the Ig supergene family (see Table 1.1) (Wawryk *et al.*, 1989).

LPAM-1 Lymphocyte adhesion to PP but not PLN HEV can be inhibited by a mAb recognising the LPAM-1 antigen (Holzmann *et al.*, 1989; Holzmann and Weissman, 1989). The structure of the α subunits of LPAM-1 and VLA-4 are almost identical (Holzman *et al.*, 1989) suggesting LPAM-1 is VLA-4 and a member of the integrin family of cell adhesion molecules (Table 1.1). The β chain of the LPAM-1 molecule appears to be antigenically distinct from other known β chains and forms a heterodimer with the α-subunit, a process which is Ca^{2+} dependent (Holzmann and Weissman, 1989).

1.7 EVIDENCE FOR CARBOHYDRATE RECOGNITION IN LYMPHOCYTE HOMING

Carbohydrate recognition systems, particularly involving SPS and glycosaminoglycans (GAGs), and their complementary lectins have been identified in many systems hence interest has grown in the role that they may play in lymphocyte migration. Some examples of this are the binding of bacteria or viruses to host cell (Ofek *et al.*, 1977; Sharon, 1987) including influenza virus recognition of cell-surface sialic acids (Wiley and Skehel, 1987) and the symbiosis between plants and nitrogen-fixing bacteria (Ho *et al.*, 1986).

Carbohydrate binding systems have been implicated in recognition between yeasts (Pierce and Ballou, 1983), slime mould aggregation (Ziska and Henderson, 1988), and reaggregation of marine sponges (Coombe *et al.*, 1987a; Coombe and Parish, 1988). Also sperm-egg adhesion (Ahuja, 1982; Bolwell *et al.*, 1980; Glabe *et al.*, 1982) and embryogenesis (Tucker, 1986; Wenzl and Sumper, 1981; Yamaguchi and Kinoshita, 1985) appear to involve SPS recognition. Adhesion of neural retina cells from embryonic chickens has also been shown to involve the heparin-binding domain of NCAM (Cole *et al.*, 1986; Cole and Glaser, 1986) and carbohydrates are involved in neuron-glial and

glial-glial adhesion (Künnenmunde *et al.*, 1988). Finally, smooth muscle cell growth and migration can be inhibited by heparin and heparin-like molecules (Castellot *et al.*, 1981; Majack and Clowes, 1984).

Carbohydrate involvement in lymphocyte homing has been indicated by three major areas of research. Firstly, the effect of modifiers of carbohydrate structure on lymphocyte migration. Secondly, the effects of lectins on lymphocyte homing and thirdly, direct inhibition of lymphocyte homing by carbohydrates.

1.7.1 Effect of Modifiers of Carbohydrate Structure on Lymphocyte Migration

A number of treatments which specifically alter carbohydrate structure on the cell surface have been used to demonstrate the direct involvement of carbohydrates in lymphocyte homing. These include treatments of cells with glycosidases, and glycosylation inhibitors (e.g. swainsonine) and periodate.

Glycosidases Gesner and Ginsburg (1964) found that thoracic duct lymphocytes which were incubated *in vitro* with glycosidases showed altered migration patterns *in vivo*. This effect was specifically blocked by inclusion of either L-fucose or N-acetyl-galactosamine respectively, during the glycosidase treatment. They therefore postulated that lymphocyte migration patterns were dependent upon the recognition of cell surface sugar molecules.

Similar results were obtained with neuraminidase (sialidase) treatment. Neuraminidase is a N-acetylneuraminylhydrolase, which cleaves the 2,3 and 2,6 glycosidic linkages between N-acetylneuraminic acids and subterminal sugars on carbohydrate sidechains. Treatment of lymphocytes with neuraminidase was found to alter their migration behaviour (Gesner *et al.*, 1969; Woodruff and

Gesner, 1969). Gesner *et al.* (1969) performed studies where radiolabelled thoracic duct lymphocytes were incubated with neuraminidase and injected intravenously into recipient rats and the resultant radioactivity in various organs measured. It was found that there was a decrease in the number of lymphocytes in the spleen and lymph nodes with a corresponding increase in the liver at 30 min and 2 h post injection, an affect which was not seen at 24 h post injection. Other studies with more highly purified preparations of neuraminidase have shown similar results (Kolb and Kolb-Bachofen, 1978; Kolb-Bachofen and Kolb, 1979). These results suggest that carbohydrate structures on the cell surface which were altered by neuraminidase treatment were essential for correct lymphocyte migration. However, these early studies with glycosidases have been severely criticised, as it has been suggested that inhibition of entry into lymphoid organs is a secondary effect resulting from sequestration of glycosidase treated lymphocytes in non-lymphoid organs due to recognition of altered cell surface carbohydrates.

The neuraminidase experiments were particularly questionable as neuraminidase treatment often resulted in exposure of subterminal galactose residues which could be recognised by a galactose-binding lectin in the liver (Ashwell and Kawasaki, 1978). In fact, the sequestration of treated lymphocytes in the liver is almost certainly due to these liver lectins. Nevertheless, more recent experiments have implicated sialic acid residues in lymphocyte HEV binding. Thus treatment of frozen sections of HEV from mouse or rat with sialidases specifically inhibited lymphocyte attachment to PLN but not PP HEV while MLN HEV showed a partial response (Rosen *et al.*, 1985). This confirmed previous ideas that MLN HEV contained receptors for both PLN and PP HEV (Stevens *et al.*, 1982). Similar results were obtained *in vivo* where injected sialidases selectively prevented subsequent *in vitro* attachment of lymphocytes to PLN HEV and also short term accumulation of lymphocytes in PLNs (Rosen *et al.*, 1989). Furthermore, the use of linkage-specific sialidases has indicated that

either α2,3 or α2,8 (but probably not α2,6) sialic acid is involved in attachments of ligands to PLN HEV (Yednock and Rosen, 1989).

Rosen *et al.* (1989) therefore suggested that sialic acid was either a direct recognition determinant of the ligand on PLN HEV or had an indirect modulatory role in HEV adherence. Differentiation between these two models may ultimately require the isolation and characterisation of the actual HEV ligands involved.

Interestingly, sialic acid may also be involved in the pathogenesis of certain bacterial and parasitic infections. Many microorganisms release sialidases into the serum possibly as a means of perturbing lymphocyte homing and thus modifying immune responsiveness (Schauer, 1985; Yednock and Rosen, 1989).

Glycosylation Inhibitors Another line of evidence which suggested direct involvement of carbohydrates in lymphocyte attachment to HEV is that afforded by treatment with the glycosylation inhibitor, swainsonine. Swainsonine treatment of lymphocytes affected lymphocyte migration to lymph nodes and the spleen (Hooghe *et al.*, 1984). Swainsonine is a potent inhibitor of mannosidase II and causes the formation of hybrid types, rather than complex types, of oligosaccharides having an oligomannosyl core characteristic of neutral oligosaccharides and one or several sequences characteristic of complex chains (reviewed by Elbein, 1987).

Periodate Sodium periodate is an oxidising agent which in dilute solution removes the terminal two carbon atoms from sialic acid and converts the alcohol group of carbon seven to the aldehyde form (van Lenten and Ashwell, 1971), it also oxidises vicinal hydroxyl groups on other carbohydrates. Zatz *et al.* (1972) demonstrated that treatment of lymphocytes with potassium periodate, under conditions selective for the oxidation of cell surface carbohydrates, inhibited the migration of lymphocytes to lymph nodes and to a lesser extent the spleen.

1.7.2 Effects of Lectins on Lymphocyte Migration

Traditionally a lectin (from Latin *legere*: to choose) is defined as a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates (Goldstein *et al.*, 1980). Lectins have been used extensively as molecular tools in biology (reviewed by Barondes, 1981; Lis and Sharon, 1986; Sharon and Lis, 1989) and have been used to determine the involvement of carbohydrates in lymphocyte migration.

In experiments performed by Schlesinger and Israël (1974) a range of lectins were tested for their ability to affect lymphocyte migration. Radiolabelled murine lymphocytes were exposed to concanavalin A (Con A), phytohaemagglutinin (PHA), a fucose binding protein or wheat germ agglutinin and then injected intravenously into syngeneic recipient mice. It was found that Con A inhibited lymphocyte migration to lymph node to a greater degree than entry into the spleen whereas PHA inhibited migration to each of these organs to a similar degree. Neither fucose binding protein or wheat germ agglutinin affected lymphocyte migration. The effect of Con A on lymphocyte migration was overcome by incubation of the Con A treated lymphocytes with α-methyl-mannopyranoside prior to injection which indicated that Con A affected lymphocyte migration by specifically binding to cell surface carbohydrates. However, as with the glycosidase studies, these experiments were criticised on the basis that lectins bound to the surface of lymphocytes, due to their multivalent nature and could modify lymphocyte migration by binding non-specifically to other cells and tissues *in vivo*.

Further studies, reviewed by Yednock and Rosen (1989) examined the effect on lymphocyte/HEV interaction of treating cryostat sections of endothelia with lectins. It was found that *Limax* agglutinin (a sialic acid-specific lectin) blocked attachment to both PP and PLN HEV whereas other lectins (Con A, wheat germ agglutinin, *Dolichus biflorus* agglutinin, *Limulus polyhemus* agglutinin, *Ricinus communis*

agglutinin and *Ulex europeus* I) had no effect. This effect was considered specific because addition of a sialylated lectin inhibitor to *Limax* agglutinin (bovine submaxillary mucin) blocked its inhibitory potential. This again demonstrated the involvement of carbohydrate structures particularly sialic acid, in lymphocyte/HEV interactions. It is interesting to note that although sialidase treatment did not affect lymphocyte binding to PP HEV (Section 1.7.1), binding was inhibited by the sialic acid-specific lectin. This could be due to sialidase-resistant forms of sialic acid (Schauer, 1982) present on PP HEV.

1.7.3 Inhibition of Lymphocyte Migration by Carbohydrates

Monosaccharides Direct evidence for the involvement of carbohydrates in lymphocyte/HEV interactions comes from the work of Stoolman and Rosen (1983). Using the *in vitro* assay (Section 1.4.2) developed by Stamper and Woodruff (1976), they tested the ability of neutral monosaccharides to inhibit the attachment of lymphocytes to rat PLN HEV. They demonstrated that L-fucose and D-mannose selectively inhibited lymphocyte/PLN HEV interactions when compared to other neutral monosaccharides. However, their results were not entirely satisfactory due to the high concentrations (75-150 mM) of monosaccharide needed to induce inhibition.

In further experiments, Stoolman *et al.* (1984), discovered more potent monosaccharide inhibitors of the lymphocyte/HEV interaction. M6P and fructose-1-phosphate (F1P) inhibited lymphocyte/PLN HEV binding by 80-90% at a concentration of 10 mM, which indicated that in terms of inhibitory activity M6P was 25-50 fold more inhibitory than D-mannose. In contrast, mannose-1-phosphate, fructose-6-phosphate, galactose-1- and 6-phosphates, glucose-1- and -6-phosphates showed little or no inhibition at 10 mM (see also Table 1.3).

Table 1.3 Inhibition of Lymphocyte Binding to High Endothelial Venules

Carbohydrate	Concentration of carbohydrate for 50% inhibition (mM)[a]
Galactose-1P	> 10
Glucose-6P	> 10
Glucose-1P	> 10
Galactose-6P	> 10
Fructose-6P	> 10
Mannose-1P	> 10
Fructose-1P	2-3
Mannose-6P	2-3
PPME	10^{-5}
Fucoidan	$<10^{-4}$

[a] Concentration of carbohydrate necessary for one half maximal inhibition of lymphocyte binding to HEV (*in vitro*).

PPME = mannose 6-phosphate-rich core mannan

Modified from Rosen and Yednock (1986); Stoolman *et al.* (1984)

To determine if M6P moieties expressed by HEV were essential for lymphocyte binding, sections of mouse PP and PLN were incubated with high concentrations of alkaline phosphatase and α-mannosidase. No subsequent inhibition of lymphocyte attachment was observed which led to the conclusion that M6P may be a structural mimic of the actual ligand on PLN HEV (Yednock and Rosen, 1989).

Phosphomannan Ester (PPME) Following these investigations another more potent inhibitor of lymphocyte attachment was discovered, the polysaccharide PPME. PPME is a M6P-rich core mannan derived from the yeast *Hansenula holstii*. It has a MW of 2.5 x 10^6 and consists exclusively of mannose residues with one in every six sugar residues being phosphorylated in the carbon-six position. Fifty percent inhibition of lymphocyte/PLN HEV binding was achieved in the rat at a concentration of 10^{-8} M (Table 1.3; Spamgrude *et al*,

1984). Similar inhibitory effects are seen with PPME in the mouse (Yednock *et al.*, 1987a) and human *in vitro* HEV assay (Stoolman *et al.*, 1987).

PPME is therefore thought to mimic a ligand for the PLN homing receptor and although it may be postulated that a carbohydrate is probably involved in the PP binding system, as yet none has been identified (Yednock and Rosen, 1989). However, PPME was found to inhibit lymphocyte attachment to lung lymph node HEV (Geoffroy *et al.*, in preparation).

To determine whether the receptor for PPME was located on the lymphocyte or the HEV surface, simple preincubation experiments were employed (Stoolman *et al.*, 1984; Stoolman and Rosen, 1983). It was found that incubation of lymphocytes but not HEV with PPME inhibited HEV binding, suggesting that PPME bound to the lymphocyte surface.

In order to demonstrate this directly, fluorescent beads were covalently coupled with PPME (Yednock *et al.*, 1987a) and then bound to lymphocytes with bead binding being quantified by flow cytometry. PPME beads bound avidly to mouse, rat and human peripheral lymphocytes with this interaction being selectively inhibited by M6P, F1P, PPME and fucoidan (Stoolman *et al.*, 1987; Yednock *et al.*, 1987a). Similarly Brandley *et al.* (1987) have employed PPME immobilised on an inert polyacrylamide surface to demonstrate that subpopulations of rat lymphocytes carry receptors for M6P and related structures. These experiments and others (Yednock *et al.*, 1987a, b) have indicated that binding of peripheral lymphocytes to PPME beads mimics the *in vitro* interaction of lymphocytes with PLN HEV seen in the Stamper and Woodruff assay (1976). Experiments were then carried out to identify the "carbohydrate-binding receptor" (Rosen and Yednock, 1986) and its relationship to other known cell surface antigens, a prime candidate being MEL-14 antigen. A series of experiments have demonstrated, in

fact, that MEL-14 is either very closely associated with or identical to the PPME receptor on lymphocytes (Yednock *et al.*, 1987b). Evidence includes the observation that the binding of PPME beads to lymphocytes was specifically blocked by MEL-14 and not by other antibodies to cell surface molecules. Also there was a high correlation between expression of PPME receptor activity and the cell-surface expression of the MEL-14 antigen on lymphoma cell lines.

Sulfated Polysaccharides (SPS) Aside from indirect evidence for the involvement of SPS in lymphocyte recirculation, such as the presence of receptors for SPS on many cell types, there is a wide body of data suggesting that SPS and their receptors may play a direct role in lymphocyte homing.

Receptors for SPS are present on a wide variety of cell types including lymphocytes (Bradbury and Parish, 1989; Parish *et al.*, 1984, 1988; Parish and Snowden, 1985; Thurn and Underhill, 1986), macrophages (Bleiberg *et al.*, 1983; Chong and Parish, 1986), endothelial cells (Glabe *et al.*, 1983b; Glimelius *et al.*, 1978), rat neurones (Vidovic *et al.*, 1986), mammary adenocarcinoma cells (Coombe *et al.*, 1987b), rat liver cells (Kjellén *et al.*, 1977), calf brain membranes (Miller and Waechter, 1984). Furthermore, there is evidence that known lymphocyte cell surface antigens such as Thy-1, Ly-5 (T-200), CD4 and CD2 interact with sulfated carbohydrates (Lederman *et al.*, 1989; Parish *et al.*, 1988a, b).

Direct evidence that SPS recognition plays a role in lymphocyte migration comes from the finding that certain SPS, such as dextran sulfate and heparin, cause leucocytosis and inhibit lymphocyte recirculation when injected into animals (Bradfield and Born, 1974; Jansen *et al.*, 1962; Sasaki and Suchi, 1967). Also, fucoidan, a sulfated polymer of L-fucose, was found to be a potent inhibitor (of similar magnitude to PPME) of lymphocyte adhesion to HEV *in vitro* (Stoolman *et al.*, 1987; Stoolman and Rosen, 1983). Furthermore, HEV can rapidly incorporate radiolabelled sulfate into a glycolipid which is

secreted and causes lymphocytes to localise at intradermal sites of injection (Andrews *et al.*, 1982, 1983).

Another very interesting line of evidence for carbohydrate involvement in lymphocyte migration is the fact that the lymphocyte homing receptors MEL-14 and CD44 may participate in carbohydrate-protein interactions (Coombe and Rider, 1989). A protein sequence in MEL-14 resembles the carbohydrate binding domain found in a number of lectins with Ca^{2+}-dependent, carbohydrate-binding activity (Lasky *et al.*, 1989). Furthermore, CD44, as previously mentioned (Section 1.6.1) contains not only a carbohydrate binding domain but also extensive glycoconjugate structures, including putative chondroitin sulfate sidechains which may be recognised by carbohydrate-binding receptors on HEV.

The most conclusive *in vivo* evidence for SPS involvement in lymphocyte recirculation comes from the work of Brenan and Parish (1986) where they demonstrated the selective effects of SPS on entry, displacement and furthermore positioning of lymphocytes within lymphoid organs. In the case of lymphocyte entry, SPS appeared to be blocking SPS receptors on the surface of endothelial cells. For example, λ-carrageenan and κ-carrageenan decreased lymphocyte entry into the spleen to 20% and 83% that of control values respectively, although they have the same polysaccharide backbone and differ only in their degree of sulfation. Also some SPS such as fucoidan and dextran sulfate, displaced lymphocytes from their normal positions in the red and white pulp of the spleen.

1.8 PROPOSED WORK

Lymphocyte migration is therefore seen as an extremely complex process involving specific interactions between ligands and receptor

molecules on the cell surface. Certain lines of evidence, such as the involvement of carbohydrates in many cell adhesion systems (Section 1.7) and the direct role they have already been seen to play in lymphocyte homing (Section 1.7.3), suggest that carbohydrates may play an intrinsic role in the process of lymphocyte recirculation.

Although the MEL-14 antigen, which is a carbohydrate-binding receptor (Section 1.6.1), has been implicated in lymph node/HEV binding, as yet molecules have not been identified on the lymphocyte surface, which are involved in splenic entry. However, evidence strongly suggests the involvement of SPS in this process (Brenan and Parish, 1986; Section 1.7.3). Furthermore nothing is known about the molecular control of lymphocyte positioning within lymphoid organs, a process which appears to be disrupted by SPS.

This study therefore focuses on the interactions between SPS ligands, initially isolated from splenic tissue, and lymphocyte receptors for these and other SPS. An attempt has then been made to isolate and characterise lymphocyte receptors and determine in part, their role in lymphocyte recirculation *in vivo*. It is hoped that this will provide some basis for future work on the role of SPS in lymphocyte recirculation.

CHAPTER 2 : MATERIALS AND METHODS

2.1 MICE

All mice were bred at the John Curtin School of Medical Research. C57BL/6 mice of either sex were used for experiments involving splenic GAGs whereas in all other experiments female mice were used from 6-20 weeks of age. C57BL/6, BALB/c and CBA mice were used for *in vivo* migration studies and BALB/c mice for maintaining cell lines.

2.2 POLYSACCHARIDES

All polysaccharides were obtained from Sigma Chemical Co. (St. Louis, MO) with the exception of dextran sulfate (MW 500,000) which was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Structural features of the polysaccharides are shown in Table 3.2. The separation of bovine lung heparin into fractions with high and low affinity for antithrombin III was performed using an antithrombin III coupled Sepharose column, according to the method of Parish *et al.* (1987). The polysaccharides were dissolved in 0.15 M NaCl at stock concentrations of 20 mg/mL, or in the case of hyaluronic acid and the carrageenans (because of their viscosity in solution) at 10 and 2 mg/mL respectively, and stored at -20°C. They were boiled for 1 min before use in binding specificity experiments.

2.3 ANTIBODIES

The mAbs used and their mode of immobilisation for immunoprecipitation experiments is listed in Table 2.1, the antibodies

Table 2.1 Monoclonal Antibodies

Antibodies	Species	Source	Antigenic determinant recognised	Immunoprecipitation method				Origin
				Sheep anti rat Ig beads	Protein A beads	Protein G beads	Sepharose 4B beads	
MEL-14	rat	cs	MEL-14	+				R. Ceredig
53.6.7	rat	cs	Ly -2	+				R. Ceredig
2.4G2	rat	cs	FcR	+				R. Ceredig
C363	rat	cs	CD3	+				R. Ceredig
53-9.2.8	rat	ascites	ThB		+			I. McKenzie
ASH 1788	mouse	ascites	Ly-5.1(T200)		+			I. McKenzie
8-6.2	mouse	ascites	Ly-15.2(LFA-1)				+	I. McKenzie
IM7.8.1	rat	ascites	Pgp-1				+	F. Lynch
ASH 1848	rat	ascites	Thy-1.2			+		I. McKenzie
A1	mouse	ascites	EL-4 43kDa protein			+		O. Kanagawa[a]

[a] Nagasawa *et al.*, 1987
cs = culture supernatant

being immobilised on one of four substrata. Purified anti-Ly-15.2 and anti-Pgp-1 antibodies were covalently coupled to CNBr-activated Sepharose-4B beads (Pharmacia) (Parish *et al.*, 1988a). A number of rat mAbs (MEL-14, anti-Ly-2, anti-FcR and anti-CD3) were adsorbed to sheep anti-rat Ig-coupled Sepharose 4B beads (Silenus laboratories, Hawthorn, Australia). The remaining antibodies were bound to either protein A-coupled Sepharose CL-4B beads (Pharmacia)(anti-ThB and anti-Ly-5.1) or recombinant protein G-coupled agarose (Genzyme Corp, Boston, MA) (anti-Thy1.2 and A1).

2.4 CELL LINES

The cell lines $RD10_S$ (gift from K. Walker, Sydney) and BCL.1 (Slavin and Strober, 1978)(Table 2.2) were passaged continuously in BALB/c mice by injecting 10^6 $RD10_S$ cells intravenously and 2 x 10^6 cells BCL.1 cells intraperitoneally. Splenic tumours were removed from $RD10_S$ and BCL.1 injected mice, 14 days and 16 days post injection, respectively. Organs were gently teased through a fine wire mesh and single cell suspensions were prepared (Section 2.5) for reinjection.

All other lymphoma lines (Table 2.2) were cultured *in vitro* in Eagle's minimum essential medium (F15; Gibco, Grand Island, NY)/ supplemented with 2.2 g/L sodium bicarbonate, 5-10% foetal calf serum (FCS) and antibiotics (penicillin 120 mg/L, streptomycin 200 mg/L, neomycin 200 mg/L).

Table 2.2 Tumour Cell Lines

Cell Line	Origin	Mouse strain	Mode of induction	Propagation	Recipient mouse strain[a]
BCL.I	B	BALB/c	spontaneous	*in vivo*	BALB/c
$RD10_S$	B	BALB/c	hybridoma	*in vivo*	BALB/c
EL-4	T	C57BL/6	benzopyrene	*in vitro*	C57BL/6
RI^+	T	C58	spontaneous	*in vitro*	CBA
RI^-	T	C58	spontaneous	*in vitro*	CBA
BL/VL3	T	C57BL/Ka	Rad LV	*in vitro*	C57BL/6
MBL-2	T	C57BL/6	Moloney LV	*in vitro*	C57BL/6
C6VL/1	T	C57BL/Ka	Rad LV	*in vitro*	C57BL/6
LSTRA	T	BALB/c	Moloney LV	*in vitro*	BALB/c
CL2-FT2	T	C57BL/6xBALB/c	Moloney LV	*in vitro*	C57BL/6
RK4.7	pre-T	C57BL/6	Abelson-Rad LV complex	*in vitro*	C57BL/6
P815	mast	DBA/2J	methylcholanthrene	*in vitro*	BALB/c

LV=leukemia virus Rad=radiation

[a] Recipient mouse strain used for *in vivo* migration studies.

2.5 PREPARATION OF CELL SUSPENSIONS

Single cell suspensions from C57BL/6 spleen were prepared in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) (Fraction V, Armour Pharmaceuticals, Eastbourne, England) as described by Parish *et al.* (1974). Suspensions were depleted of red and dead cells by Isopaque-Ficoll separation (Davidson and Parish, 1975).

Blood was collected from sheep in Alsever's solution and the red cells washed four times in 20 volumes of 0.15 M NaCl by centrifugation (600*g*, 5 min, 20°C) just before use.

2.6 COUPLING OF POLYSACCHARIDES TO ERYTHROCYTES

For rosetting assays, polysaccharides were coupled to sheep red blood cells (SRBC) via $CrCl_3$ using a method described in detail by Parish and Snowden (1985). Briefly, to 0.9 mL of 0.15 M NaCl was added 0.05 mL of packed SRBC and 0.4 mg of polysaccharide dissolved in 0.15 M NaCl. After mixing, 0.1 mL of 0.1% $CrCl_3$ in 0.15 M NaCl was added with constant shaking. The mixture was left to react at room temperature (RT) for 5 min and the reaction was stopped by addition of 4.0 mL of PBS. The coupled SRBC were pelleted by centrifugation (400*g*, 5 min, 20°C) and washed with 5.0 mL of PBS. The final SRBC pellet was resuspended in 5.0 mL PBS/0.1% BSA/0.1% NaN_3 and stored at 4°C. PS-coupled (polysaccharide-coupled) SRBC were used within 2 days of coupling.

2.7 ROSETTING ASSAYS

The rosetting of murine lymphocytes (splenocytes and thymocytes) with PS-coupled SRBC was carried out in 96-well, round-bottomed microplates (Linbro Chemical Co., New Haven, Conn.) as described previously (Parish and Snowden, 1985). To 25 µL/well of lymphocytes (4 x 10^6/mL in ice-cold PBS/0.1% BSA) was added an equal volume of 1% (v/v) PS-coupled SRBC in the same medium. The cells were mixed by gentle vortexing of the microplate and pelleted by centrifugation (200*g*, 1 min, 4°C). The pellet was then incubated on ice for at least 30 min before being gently resuspended with a Pasteur pipette. Methyl violet staining solution, 0.05% (w/v) in PBS, was prepared immediately prior to use by dilution of a stock solution of 1% (w/v) methyl violet in double distilled water (DDW). Then 50 µL of the staining solution was added to each well. Each sample was transferred to a haemocytometer slide for counting and the percentage of rosette-forming cells assessed by scanning 100 lymphocytes. Any lymphocyte that bound four or more red cells was classified as a rosette.

2.8 RADIOLABELLING OF CELL SURFACES AND PREPARATION OF CELL LYSATES

Lymphocytes and lymphoma cell lines were cell surface labelled with ^{125}I using the lactoperoxidase catalyzed method (Parish *et al.*, 1978) and a detergent lysate prepared by resuspending the labelled cells to a concentration of 4 x 10^7/mL in 0.15 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.5% Triton X-100 (TTS) containing 300 KIU/mL of aprotinin (Sigma). After incubation (30 min, 4°C) cell debris and nuclei were pelleted by centrifugation (28,000*g*, 20min, 4°C).

2.9 COUPLING POLYSACCHARIDES TO CARBOXYMETHYL CELLULOSE

Polysaccharides were coupled to carboxymethyl cellulose (CMC) based on a method reported earlier (Parish *et al.*, 1988a). Precycled fibrous CMC (CM-23, Whatman) was poured into a 10 mL Econo-Column (Bio-Rad, Richmond, CA) until approximately 3 mL of packed fibres was obtained. The column was equilibrated with 20 mL of 0.15 M NaCl and then 10 mL of 1% (w/v) $CrCl_3$, prepared in 0.15 M NaCl (Parish and McKenzie, 1978) was run into the column. The $CrCl_3$ was left to react with the CMC for 1 h at RT and then unreacted $CrCl_3$ was removed by washing with 20 mL of 0.15 M NaCl. A solution containing polysaccharides at 5 mg/mL in 0.15 M NaCl was run into the column (4 mL/column) and left to react for 16-18 h at RT. The column was then washed with 10 mL of 0.15 M NaCl and 20 mL of PBS, fibres removed from the column and stored at 4°C containing 0.1% NaN_3 for up to 12 months. It has been estimated that approx. 1 mg of polysaccharide was bound per mL of packed CMC fibres (Parish *et al.*, 1988a).

2.10 BINDING OF LYSATES TO AFFINITY COLUMNS

For binding of lymphocyte lysates to PS-coupled CMC fibres, 50 µL of packed fibres were transferred to 1.5 mL eppendorf test tubes, washed (three times) by centrifugation with 1.0 mL TTS and reacted with 0.2 mL of ^{125}I-labelled lymphocyte lysate (10^7 cell equivalents) for 1 h at 4°C on a rotator (Parish *et al.*, 1988 a, b). The fibres were then washed five times with TTS and the final sixth wash with 0.15 M NaCl, 0.05 M Tris-HCl pH 8.0 (TS). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, samples were

resuspended in 50 µL of either reduced or non-reduced sample buffer and boiled in a water bath for 5 min prior to analysis.

In some experiments, following washing the fibres six times with TTS, bound material was eluted from the fibres with 2 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.5% Triton X-100. The eluate was desalted by passage through a PD-10 column (Pharmacia) equilibrated with TTS and the resultant ^{125}I-labelled fractions containing polysaccharide-binding proteins were pooled.

For isolation of Con A, gelatin or lentil lectin binding material, ^{125}I-labelled splenocyte lysates (prepared as described above) were reacted with 50 µL of packed, TTS equilibrated, Con A-Sepharose 4B (Pharmacia), gelatin-Sepharose 4B (Pharmacia) or lentil lectin-Sepharose 4B beads (Pharmacia), for 1 h at 4°C on a rotator. The beads were then washed in TTS as for the CMC fibres in TTS and analysed by SDS-PAGE (Section 2.21) or isoelectric focussing (Section 2.22).

2.11 RELEASE OF HEPARIN-BINDING MOLECULES FROM CELLS

In order to release certain heparin-binding molecules from lymphocytes ^{125}I-labelled lymphocytes (10^7 cells) were resuspended at 4°C in 0.25 mL of solutions of PBS containing 2.5% Tween 40, 2.5 mM iodoacetamide, 1% (w/v) aprotinin (Standring and Williams, 1978) for 1 h; 2.5 M KCl for 5 min; 1 mg/mL RGDS (Arg-Gly-Asp-Ser; Peptide Technology Ltd., Sydney) for 30 min or 10 mM inositol hexaphosphate (phytic acid, Sigma) (Ishihara *et al.*, 1987) for 30 min. The cells were then pelleted by centrifugation (300*g*, 5 min, 4°C) and solubilised as described earlier with TTS and aprotinin (Section 2.8). Supernatants were desalted by passage through a PD-10 column

equilibrated with TTS and the resultant ^{125}I-labelled fractions which contained the released cell-surface proteins pooled.

Lysates of cell pellets and cell supernatants were bound to heparin-CMC fibres (Section 2.10) and bound material analysed by SDS-PAGE. Alternatively, inositol hexaphosphate released proteins were acetone precipitated directly and then analysed by SDS-PAGE (Section 2.21)

^{125}I-splenocytes were also treated with solutions of PBS containing 0.31-10 mM inositol hexaphosphate; 5 mM DL-myo-inositol 1-monophosphate (1-MP; Sigma); 5 mM myo-inositol 2-monophosphate (2-MP; Sigma); and 5 mM myo-inositol hexasulfate (HS; Sigma); and PBS alone by a modified method of Ishihara *et al.* (1987). Released proteins were analysed by SDS-PAGE.

2.12 REASSOCIATION OF HEPARIN-BINDING MOLECULES WITH LYMPHOCYTES

In some experiments ^{125}I-labelled heparin-binding molecules released from lymphocytes by 5 mM inositol hexaphosphate treatment were allowed to reassociate with lymphocytes. The inositol hexaphosphate eluted molecules in PBS (10^7 cell equivalents in 1 mL) were allowed to bind to splenocytes (10^7 cells; 1h, 4^{o}C) pretreated with 5 mM inositol hexaphosphate.

In order to detect low affinity binding of labelled molecules to cells, the cell eluate mixture (1 mL) was layered on 0.3 mL dibutyl phthalate (Selby, Sydney) in an eppendorf tube and centrifuged (28,000*g*, 30 min). The supernatant and oil were removed and the cell pellet resuspended in 50 µL of reduced sample buffer for SDS-PAGE analysis.

In inhibition of reassociation experiments, ^{125}I-labelled, inositol hexaphosphate released molecules were incubated with inositol hexaphosphate pretreated lymphocytes as described above in the presence of 0.63-10.0 mM inositol hexaphosphate, 5 mM 1-MP, 5 mM 2-MP or 5 mM HS. Following washing of cells by centrifugation through oil as described above, the cell-bound ^{125}I was counted and the percent of the control binding calculated, where control (PBS) binding in absence of inhibitor was taken as 100%.

2.13 ISOLATION OF SPLENIC-GAGS

The major steps in the isolation of murine splenic GAGs from murine spleen are depicted in Figure 2.1.

Murine C57BL/6 spleens (about 600-1200) were collected and stored frozen in PBS. Initially spleens were defrosted and drained of PBS and the packed volume measured. Half a volume of ice cold DDW was added and the mixture blended on ice in an Ato-mix blender (MSE, London, England) on the maximum setting for 1 min. Blended spleens were added gradually to 10 volumes of ice cold acetone with vigorous stirring, the precipitate allowed to settle on ice for 15 min and the acetone decanted. Another 3 volumes of ice cold acetone was added, the mixture stirred vigorously for 5 min and the precipitate allowed to settle for 15 min. The final acetone wash was repeated and the acetone removed by filtration using a large Buchner funnel, under suction. The precipitate was then transferred to a glass tray and spread out to dry overnight (with occasional stirring) at RT in a fume hood. The dried precipitate was weighed and ground with a mortar and pestle to a fine powder. The powder was suspended in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1% NaN_3 (10 mL buffer/g of powder), pronase (15 mg/g powder, protease from *Streptomyces*

Mouse spleens (600-1200)

↓

Delipidate by acetone extraction

↓

Pronase digest acetone powder for 3 days at 37°C

↓

Adjust to 0.5M NaCl and CPC precipitate GAGs

↓

Remove CPC by ethanol precipitation

↓

Precipitate contaminating DNA and peptides with TCA and dialyse supernatant against distilled water

↓

Precipitate GAGs from supernatant with CPC and remove CPC by ethanol precipitation

↓

'Crude' splenic-GAG preparation.

Figure 2.1 Procedure for Isolating Murine Splenic-GAGs from Murine Spleen

griseus , Sigma) added and the mixture incubated at 37°C for 3 days with occasional shaking.

Following pronase treatment undigested material was pelleted by centrifugation (2500*g*, 10 min), the supernatant harvested and adjusted to 0.5 M NaCl by addition of 2 M NaCl and GAGs isolated by 5% (w/v) cetylpyridinium chloride (CPC; Stansens, Kingsgrove, Australia) and ethanol precipitation as previously described (Rodén *et al.*, 1972). The ethanol precipitated material was redissolved in DDW and contaminating DNA and peptides precipitated overnight at 4°C by the addition of 0.2 volumes of ice cold 50% (w/v) trichloroacetic acid (TCA).

The precipitate was pelleted by centrifugation (16000 *g*, 15 min, 4°C) and the supernatant dialysed for 2 h against DDW, the dialysate being changed every 30 min. The dialysed solution was adjusted to 0.05 M Tris-HCl buffer, pH 8.0, and 0.15 M NaCl and the GAGs reprecipitated by CPC and ethanol (Rodén *et al.*, 1972). The final GAG precipitate was dissolved in DDW and the amount of SPS present determined by the dimethylmethylene blue assay (Farndale *et al.*, 1986) using chondroitin-4-sulfate as a standard. The protein content of the GAG preparation was determined by the Coomassie Blue procedure (Rylatt and Parish, 1982) and the DNA/RNA content assessed by absorbance at 260/280 nm using a DMS 100 Varian spectrophotometer.

2.14 ENZYMATIC DIGESTION OF GAGS

For chondroitinase digestions, GAGs were dissolved in 0.1 mL of 0.05 M sodium acetate, 0.05 M Tris-HCl buffer, pH 8.0, containing 1.25 U/mL of either chondroitinase ABC (from *Proteus vulgaris*, Sigma) or chondroitinase AC (from *Arthrobacter aurescens*, Sigma), 0.1% (w/v) sodium azide added and the solutions incubated overnight at 37°C. In

the case of RNase and DNase, GAGs were digested with 20-25,000 U/mL of enzyme for 30 min at 37°C in PBS or 0.1 M NaCl, 0.1M $MgCl_2$ and 0.01M Tris-HCl buffer, pH 7.5 respectively. For binding experiments solutions were then boiled for 5 min to destroy the enzyme. Control digests did not contain the enzyme. For nitrous acid cleavage of radiolabelled GAGs 9 volumes of 0.24 M $NaNO_2$ in 1.8 M acetic acid was added and the mixture incubated overnight at RT (Lindahl *et al.*, 1973). The mixture was then either dialysed against DDW (2 h, RT, with 30 min changes in DDW), lyophilised and redissolved in SDS-PAGE sample buffer (Laemmli, 1970) or dialysed against PBS if the preparation was radiolabelled and was to be used for subsequent splenocyte binding assays.

Chondroitinase digestion of unlabelled GAGs was monitored by a previously described dimethylmethylene blue assay (Farndale *et al.*, 1986), although decrease in absorbance at 600 nm was used to quantify GAGs rather than increase in absorbance at 525 nm as reported earlier. The method was automated and miniaturised by performing the assays in 96-well, flat bottom microtitre plates (Nunclon Delta, Roskilde, Denmark) and reading absorbance values on a Dynatech MR600 microplate reader (Dynatech Labs, Alexandria, Va). Digestion of the ^{125}I-labelled GAG preparation by chondroitinases was monitored by passage of the digested sample through a PD10 column and measuring ^{125}I-labelled undigested material in the excluded peak. This elution profile was compared with that obtained with an untreated sample of labelled GAGs.

2.15 PREPARATION OF RADIOLABELLED GAGS

GAGs were fluoresceinated and radiolabelled with ^{125}I as described by Glabe *et al.* (1983a). Briefly, the procedure entailed CNBr activation of the GAGs, coupling the activated GAGs with fluoresceinamine and subsequent ^{125}I-labelling of the attached

fluorescein moieties. Unincorporated ^{125}I was removed by dialysing the radiolabelled preparations for 2 h at RT against PBS.

2.16 BINDING OF RADIOLABELLED GAGS TO CELLS

Binding assays were performed in either 1.0 x 7.6 cm plastic centrifuge tubes (3DT tubes, Disposable Products, Adelaide, Australia) or in the case of larger experiments and where timing was critical, 96-well, round bottom microplates were used. The reaction mixture consisted of 100 µL of ^{125}I-splenic-GAGs in PBS containing 0.1% BSA to which 100 µL (4×10^6) of splenocytes was added and the cells left to react on ice for 60 min with occasional shaking. Tube assay samples were washed three times by centrifugation (300 *g*, 5 min, 4°C) with 1 mL/tube of ice cold PBS/0.1% BSA whereas in the microplate assay, cells were washed with PBS/0.1% BSA using a Titertek cell harvester (Flow Laboratories Ltd., Irvine, Scotland). Air dried filter paper discs were collected and cell bound ^{125}I counted. To correct for nonspecific binding, control binding assays were performed in the presence of a 50-150 fold excess of unlabelled heparin (shown to inhibit binding of labelled splenic-GAGs). In binding-inhibition experiments 50 µL aliquots of inhibitor (6.25-800 µg/mL) were placed in microplates, a constant amount of ^{125}I-splenic GAGs (50 µL, 20 µg/mL) added followed by 4×10^6 splenocytes in 100 µL and the standard binding assay performed.

2.17 ION EXCHANGE FRACTIONATION OF GAGS

C57BL/6 splenic-GAGs were prepared, iodinated (Section 2.8) and dialysed 2.5 h against either 0.25 M NaCl, 0.01 M Tris-HCl buffer, pH 7.0 (ready for ion exchange fractionation) or chondroitinase treatment

buffer (see above). The latter sample was chondroitinase ABC digested overnight at 37°C and degradation products removed by passage of the sample through a PD10 column equilibrated with 0.25 M NaCl, 0.01 M Tris-HCl buffer, pH 7.0.

A DEAE Sepharose CL6B column (41.5 x 1.8 cm, Pharmacia) was equilibrated with 0.25 M NaCl, 0.01 M Tris-HCl buffer, pH 7.0, the sample applied and the column washed with 50 mL of equilibration buffer prior to elution of material from the column with a 200 mL linear 0.25-1.0 M NaCl gradient, buffered with 0.01 M Tris-HCl, pH 7.0. The column flow rate was 10.2 mL/h and 2 mL fractions were collected and assayed for NaCl content by refractometry and counted for radioactivity. Groups of fractions (20 per group) were pooled (Pools I-VII), dialysed overnight against DDW, lyophilised and redissolved in 2 mL PBS/0.1% BSA.

2.18 BIOTINYLATION OF GAGS

Splenic-GAGs (2 mg in 1.0 mL DDW) were CNBr activated by the addition of 0.2 mL of CNBr (50 mg/mL in DDW) and the mixture left to react for 5 min at RT, the pH being maintained at 11.0 by the addition of 1 M NaOH. Unreacted CNBr was removed by passage of the splenic-GAGs through a PD-10 desalting column equilibrated with 0.2 M borate buffer, pH 8.0. The excluded peak was collected (2.0 mL), 4 mg of biocytin (N-ε-biotinyl-L-lysine) added and the mixture left to react at RT overnight. Biotinylated GAGs were separated from free biocytin by passage through another PD-10 column and collection of the excluded peak (3.0 mL). Based on a MW of 20 kDa, each GAG molecule was conjugated with 4-5 biotin molecules.

2.19 IMMUNOFLUORESCENT FLOW CYTOMETRY

Binding of biotinylated splenic-GAGs to murine splenocytes was quantified by flow microfluorometry using an indirect immunofluorescence technique similar to that described by Berman and Basch (1980). Briefly, 50 µL of splenocytes (10^7/mL) were added to 50 µL of biotinylated GAGs (1.6-200 µg/mL) in PBS/0.1% BSA and the mixture incubated on ice for 60 min. Following washing in PBS/0.1% BSA the cells were incubated with a second step avidin-fluorescein conjugate (Becton Dickinson, Immunocytometry Systems, Mountain View, CA; 1:20 dilution), a third step rabbit anti-avidin antiserum (Sigma; 1:250 dilution) and a fourth step fluorescein conjugated sheep $(Fab')_2$ anti-rabbit Ig (Silenus; 1:10 dilution); the cells were washed twice with F15/5% FCS between each incubation step. Labelled cells were analysed on a Becton-Dickinson FACS IV (Becton Dickinson) with appropriate controls for each incubation step.

2.20 IMMUNOPRECIPITATIONS

Immunoprecipitations were performed with 10-15 µL of packed antibody-coupled beads, different mAbs being immobilised to different solid supports as listed in Table 2.1.

To reduce binding of ^{125}I-labelled mouse Ig to sheep anti-rat beads, radiolabelled murine lymphocyte lysates were incubated overnight with uncoupled sheep anti-rat Ig beads (0.25 mL lysate/10 µL beads). In addition, binding of radiolabelled lysate to mAb-coupled beads was performed in the presence of 1% heat-inactivated mouse serum in TTS (10 µL serum/10 µL beads).

2.21 GEL ELECTROPHORESIS

^{125}I-labelled proteins were precipitated with acetone from solutions for gel electrophoresis by mixing in an eppendorf tube 0.2 mL ^{125}I-labelled protein solution in TTS and 5 µL PBS/0.1% BSA (5 µg carrier BSA) with 1 mL of ice cold acetone. After incubation on ice for 5 min the protein precipitate was pelleted by centrifugation (28,000*g*, 5 min, 4°C) and the pellet resuspended in 50 µL of reduced sample buffer for SDS-PAGE analysis.

Pooled fractions of radiolabelled GAGs from ion exchange fractionations were analysed by one dimensional 10% and 15% SDS-PAGE according to the method of Laemmli (1970). In all other experiments samples were analysed by 8-18% SDS-PAGE, the samples were prepared in 50 µL of either reduced or non-reduced sample buffer and were boiled 5 min before gel loading. Gels were stained with Coomassie blue, destained to localise added MW markers (Bio-Rad Laboratories, Richmond, CA), then dried for autoradiography and MW markers identified with radioactive ($^{35}SO_4^{2-}$) ink. The MW markers used were myosin MW 200,000; *E.coli* β-galactosidase MW 116,250; rabbit muscle phosphorylase b MW 97,400; BSA MW 66,200; ovalbumin MW 42,699; bovine carbonic anhydrase MW 31,000; soybean trypsin inhibitor MW 21,500 and hen egg white lysozyme MW 14,400. Dried gels were autoradiographed at -70°C on Kodak XAR-5 film using Cronex intensifying screens and in some experiments scanned using a LKB UltroScan XL Laser Densitometer (Bromma, Sweden).

2.22 ISOELECTRIC FOCUSSING

To determine the isoelectric point of the 90 kDa protein, 2 x 10^7 C57BL/6 murine splenocytes were cell-surface labelled with ^{125}I, the labelled cells lysed and the heparin-binding molecules in the lysate prepared (Section 2.8, 2.10). The 90 kDa protein was then further purified by binding the heparin eluate to lentil lectin-Sepharose 4B beads (Section 2.10), and ^{125}I-labelled material was eluted with 20 µL of 0.2 M α-methyl-D-mannoside (Sigma). The isoelectric point of the lentil lectin eluate was determined by the Phast Gel System using a homogeneous polyacrylamide gel (43 x 50 x 0.35 mm) containing Pharmalyte carrier ampholytes (22 µmol/mL pH unit) which was pre-run at 2000 volts, 3.5 W for 75 volt hours to establish a linear pH gradient in the range 3-9. One microlitre samples of ^{125}I-labelled lysate were loaded electrophoretically from a plastic comb applicator at 2000 volts, 3.5 W for 420 volt hours at 15°C. The samples and pI markers were focussed at 2000 volts, 3.5 W for 410 volt hours over a separation length of 37 mm. The pI markers used were amyloglucosidase pI 3.5; soybean trypsin inhibitor, pI 4.55; β-lactoglobulin A, pI 5.20; bovine carbonic anhydrase B, pI 5.85; human carbonic anhydrase, pI 6.55; horse myoglobin, pI 6.85 and 7.35; lentil lectin, pI 8.15, 8.45 and 8.65; and trypsinogen, pI 9.30. Protein bands were detected by Coomassie Blue staining, and the gel was autoradiographed for ^{125}I-labelled proteins.

2.23 ANALYSIS OF *IN VIVO* LYMPHOCYTE MIGRATION

For lymphocyte migration studies the method used to label fluorescently cells with bis-benzimide trihydrochloride, Hoechst 33342 (H33342, Sigma) was based on the procedure described by Brenan and Parish (1984) and Brenan *et al.* (1985). H33342 was

prepared as a 600 μg/mL stock solution in DDW and stored at 4°C. Lymphocytes or lymphoma cell lines at 5 x 10^7/mL in PBS were incubated with 6 μg/mL H33342 for 15 min at 37°C. Cells were washed twice by centrifugation (300 *g*, 5 min, 4°C) and resuspended to a concentration of 4 x 10^7 cells/mL in PBS, 0.5 mL (2 x 10^7 cells) of cells being injected intravenously into recipient mice of the appropriate strain (Table 2.2).

Spleen and lymphoid organs were removed 2 h post injection and cell suspensions prepared from whole organs by processing the cells through a fine wire mesh. The suspension was then declumped, resuspended in a fixed volume of media, and H33342-labelled cells counted in a haemocytometer chamber using an Olympus microscope (Model BHT) with a HBO (100 W) mercury vapour lamp for epi-illumination and appropriate barrier and filter combinations for H33342 (364 nm excitation and >435 nm emission). The percentage of cells entering an organ was calculated relative to splenocyte entry which was taken as 100%.

Alternatively, for positioning studies spleens were placed directly into 4% paraformaldehyde in PBS for overnight fixation. Topographical localisation of fluorescent cells was visualised by a modification of the method of Brenan and Parish (1986). Fixed murine spleens were sectioned approximately in half, placed on nonfluorescent silicone rubber (Silastic, Dow Corning, Midland, MI) and viewed and photographed under a Olympus microscope.

CHAPTER 3 : ISOLATION AND CHARACTERISATION OF ENDOGENOUS SPLENIC-GLYCOSAMINOGLYCANS

3.1 INTRODUCTION

The molecular and cellular basis of lymphocyte migration and subsequent positioning within lymphoid tissues is not well understood. Lymphocytes exhibit defined migration patterns, showing preferences for certain lymphoid tissues (Butcher *et al.*, 1980; Chin and Hay, 1984; Stevens *et al.*, 1982) and sites within these tissues (De Sousa, 1981). Specific cellular interactions have been implicated in this phenomenon, the most notable being the binding of lymphocytes to HEV in lymph nodes (Butcher *et al.*, 1979, 1980; Chin *et al.*, 1982; Gallatin *et al.*, 1983; Woodruff and Clarke, 1987), the reaction of lymphocytes with macrophages in the marginal zones of the spleen (Humphrey, 1980) and the interaction of lymphocytes with interdigitating cells in the T-dependent regions of lymphoid organs (Inaba and Steinman, 1986).

Recent studies in a range of biological systems have suggested that SPS recognition plays a key role in a range of cell-adhesion systems (Section 1.7) such as sponge cell aggregation (Coombe *et al.*, 1987a), neuronal cell-cell adhesion (Cole *et al.*, 1986), sperm-egg adhesion (Ahuja, 1982; Bolwell *et al.*, 1980; Glabe *et al.*, 1982) and embryogenesis (Tucker, 1986; Wenzl and Sumper, 1981; Yamaguchi and Kinoshita, 1985). Furthermore, receptors for SPS are present on many cell types (Section 1.7.3) including macrophages (Bleiberg *et al.*, 1983; Chong and Parish, 1986), endothelial cells (Glabe *et al.*, 1983b; Glimelius *et al.*, 1978), mammary adenocarcinoma cells (Coombe *et al.*, 1987b), and lymphocytes (Parish *et al.*, 1984, 1988; Parish and Snowden, 1985; Thurn and Underhill, 1986). Thus it seems possible that SPS recognition may play a role in the migration and positioning of lymphocytes within lymphoid tissues.

Some experimental data, in fact, supports this view (Section 1.7.3), such as the inhibition of lymphocyte recirculation and leukocytosis caused by injection of certain SPS (Bradfield and Born, 1974; Jansen *et*

al., 1962; Sasaki and Suchi, 1967) and inhibition of lymphocyte binding to HEV *in vitro* by fucoidan (Stoolman and Rosen, 1983). Different SPS can also selectively modify entry and positioning of lymphocyte subpopulations within lymphoid organs (Brenan and Parish, 1986), possibly by blocking receptors on lymphocytes for SPS.

If SPS recognition plays such a key role in lymphocyte migration and positioning, one would predict that endogenous SPS isolated from lymphoid tissue should bind to lymphocytes and in some way effect their recirculation behaviour. This chapter describes attempts to isolate and characterise the endogenous sulfated GAGs present in murine spleen and determine whether murine splenic lymphocytes carry cell surface receptors for any of these GAGs.

3.2 RESULTS

3.2.1 ISOLATION OF SPLENIC-GAGS

Earlier studies have shown that murine lymphocytes carry cell surface receptors for SPS (Parish *et al.*, 1984; 1988; Parish and Snowden, 1985; Thurn and Underhill, 1986). To determine if there are endogenous SPS in murine spleen that bind to murine splenic lymphocytes, GAGs were first isolated from murine spleens (Section 2.13; Figure 2.1). Briefly, homogenised spleens were delipidated by acetone extraction, deproteinised and GAG sidechains released by pronase digestion and then sulfated-GAGs precipitated by CPC. A high NaCl concentration (0.5M) was used during CPC precipitation to ensure that only sulfated GAGs were precipitated (i.e. no hyaluronic acid contaminated the preparation) and to minimise DNA contamination. TCA precipitation was then used to remove residual contaminating DNA and peptides and finally, the 'crude' splenic-GAGs were precipitated with CPC and ethanol (Rodén *et al.*, 1972). With C57BL/6

mice, 1000 spleens produced 21 g of acetone extracted powder which yielded 6.5 mg of 'crude' splenic-GAGs. Based on protein and DNA/RNA assays, the GAG preparation contained no detectable protein and contained less than 1% RNA and DNA.

The GAG composition of the 'crude' splenic-GAG preparation was assessed by chondroitinase ABC and AC digestion and nitrous acid treatment (Section 2.14). It was found that 84.5 $\pm$ 16% of the preparation was chondroitinase ABC sensitive and 60.9 $\pm$ 22% chondroitinase AC sensitive (Table 3.1). Nitrous acid treatment, which selectively degrades heparan sulfate/heparin chains, destroyed <15% of the 'crude' splenic-GAG preparation. Since fluoresceinated and ^{125}I-labelled splenic-GAGs were used in subsequent binding studies (Section 3.2.2; 3.2.3) it was important to determine the composition of the labelled GAG preparation. It was found (Table 3.1) that the majority of the labelled GAGs were chondroitinase ABC sensitive although substantially less was chondroitinase AC sensitive when compared with the unlabelled material. This suggests that dermatan sulfate in the preparation may have been more efficiently fluoresceinated than the chondroitin sulfates. Similarly, there appeared to be a somewhat higher proportion of nitrous acid sensitive GAGs in the labelled preparation.

3.2.2 Binding of Splenic-GAGs to Splenocytes

The isolated splenic-GAGs were radiolabelled with ^{125}I (Section 2.15) and their binding to C57BL/6 splenocytes assessed (Section 2.16). Figure 3.1 depicts a typical binding curve which is indicative of saturable binding. The data presented was corrected for non-specific binding by the inclusion of a 50-fold excess of unlabelled heparin as binding-inhibition experiments (Section 3.2.3) showed that heparin was a potent inhibitor of splenic-GAG binding. At all concentrations non-specific binding represented less than 15% of specific binding. The data when applied to a Scatchard plot (Figure 3.1b) approached

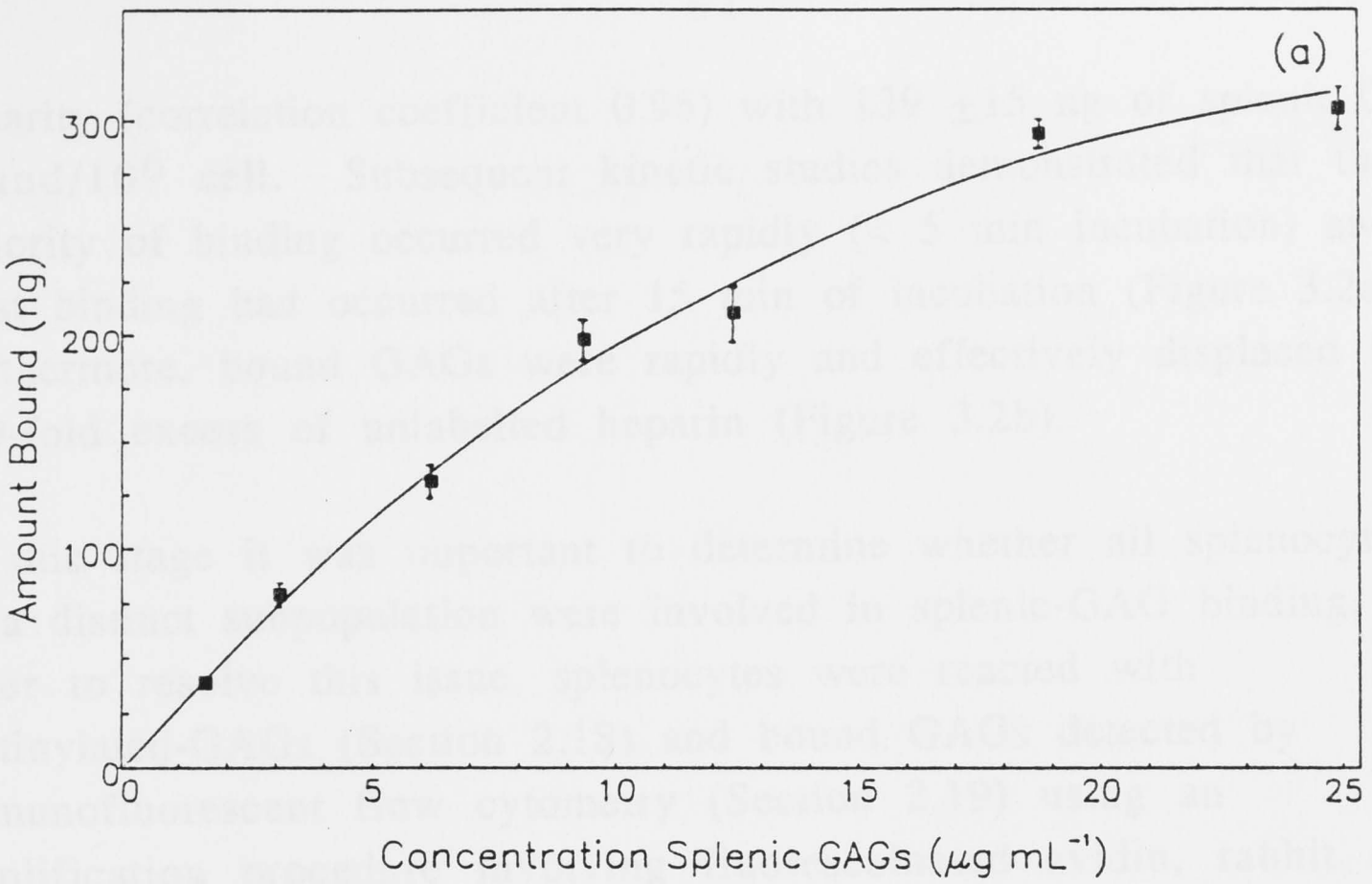
Table 3.1 Effect of Different Degradative Procedures on Binding of Splenic-GAGs to Splenic Lymphocytes

Treatment	Degradation (%) Unlabelled GAGs [a]	Labelled GAGs [b]	Binding of labelled GAGs to splenocytes (%) [c]
Nil	-	-	100 ± 3.0
Chondroitinase ABC	84.5 ± 16	60.3	130 ± 4.3
Chondroitinase AC	60.9 ± 22	18.4	96 ± 3.7
Nitrous acid	<15	30.9	56 ± 3.7

[a] Degradation of unlabelled GAGs monitored by a dimethylmethylene blue assay. Each data point is the mean of three replicates ± standard errors.

[b] Represents ^{125}I-label associated with undegraded GAGs. Each data point represents a single determination.

[c] Mean of three to six data points ± standard errors using 10 μg/ml of labelled GAGs.



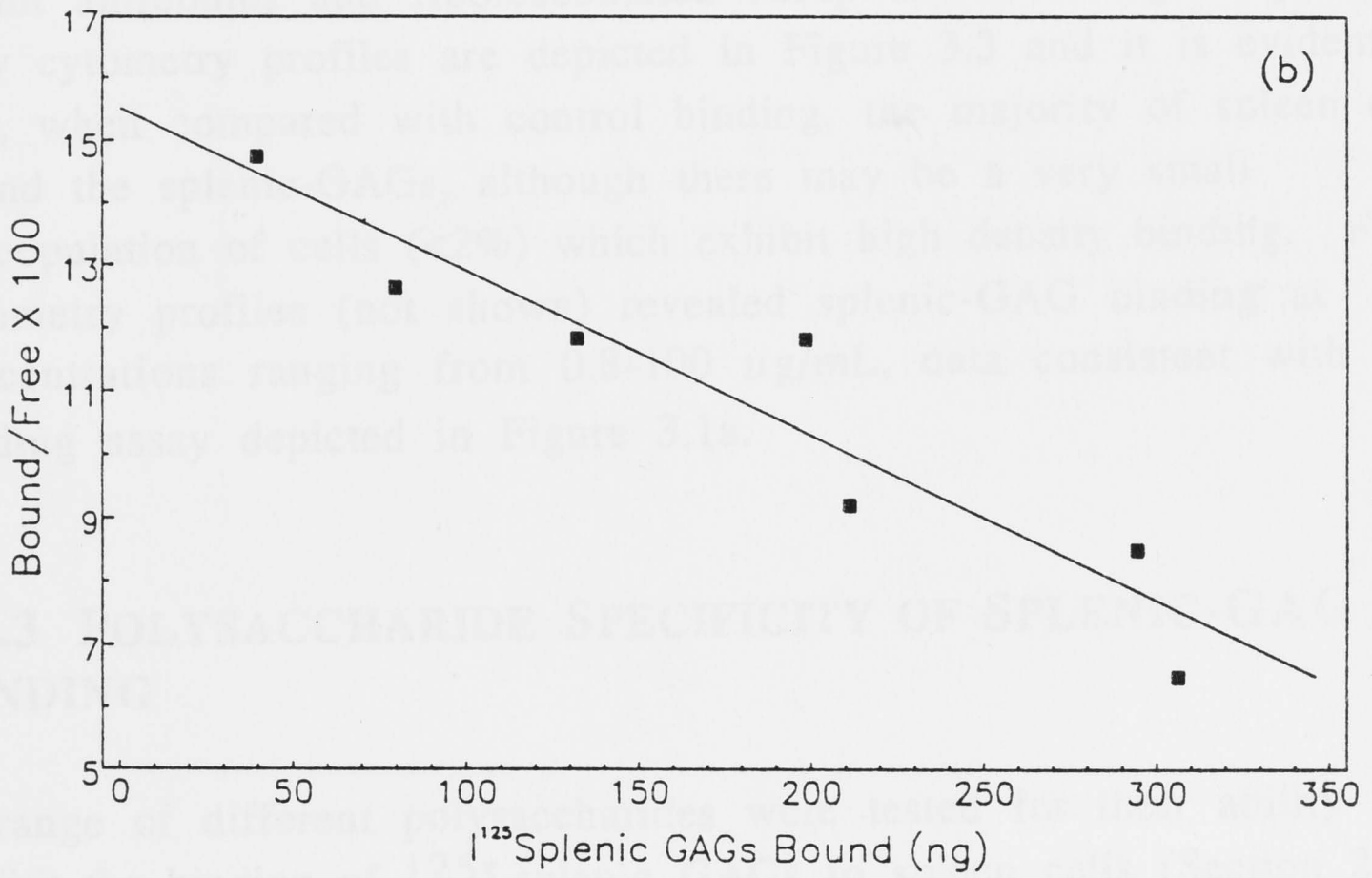


Figure 3.1 Analysis of Binding of Radiolabelled Murine Splenic-GAGs to Murine Splenocytes.

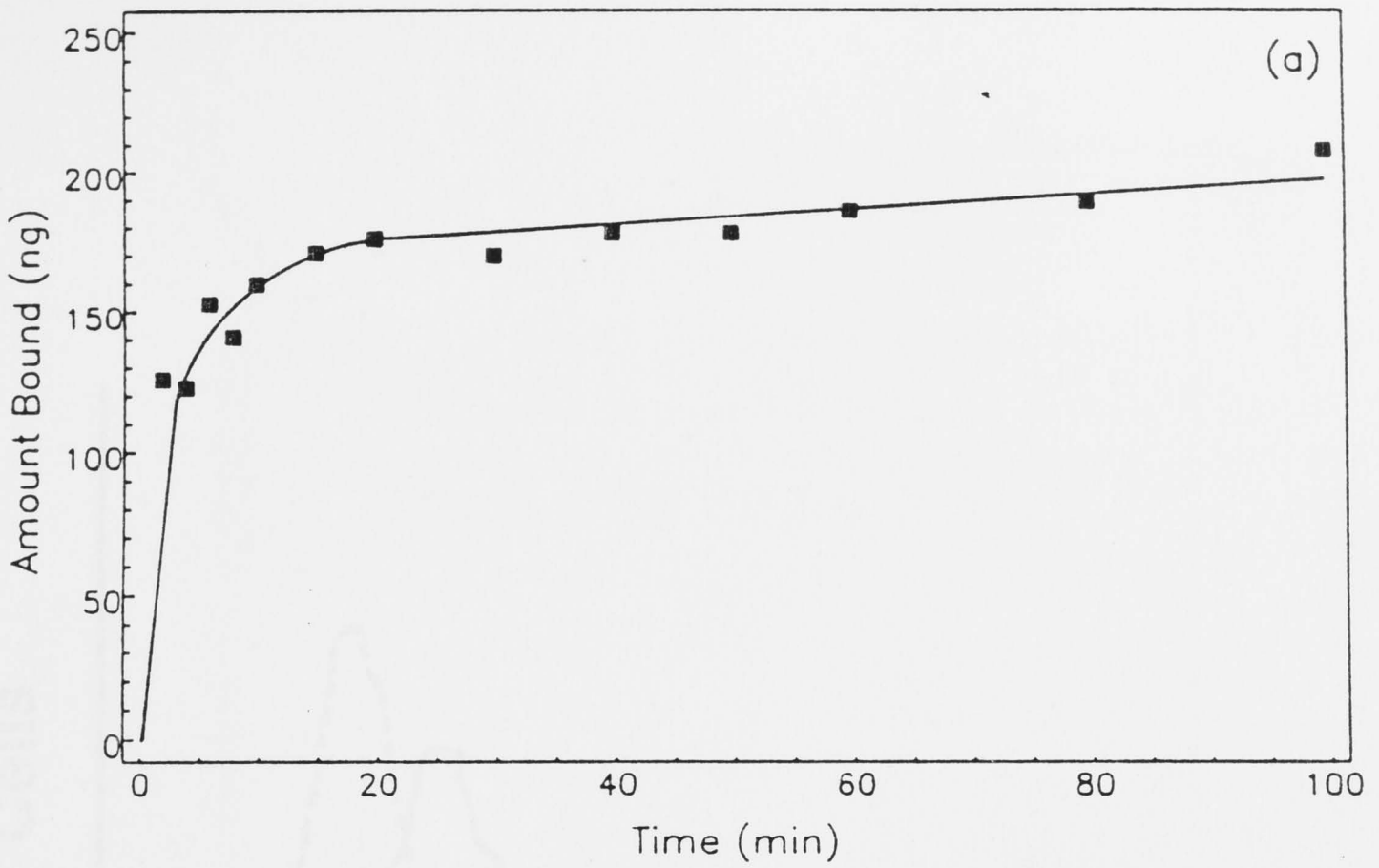
(a) Concentration dependence of binding of ^{125}I-splenic GAGs to splenocytes. The data shown have been corrected for nonspecific binding as defined by the amount of radioactivity associated with the splenocytes in the presence of a 50-fold excess of unlabelled heparin. Washing of cells was performed by centrifugation. The data shown are the mean of five replicates $\pm$ standard errors of means. (b) Scatchard analysis of splenic-GAG binding data (correlation coefficient = 0.96)

linearity (correlation coefficient 0.96) with 139 ±15 ng of splenic-GAGs bound/10^6 cell. Subsequent kinetic studies demonstrated that the majority of binding occurred very rapidly (< 5 min incubation) and most binding had occurred after 15 min of incubation (Figure 3.2a). Furthermore, bound GAGs were rapidly and effectively displaced by a 150-fold excess of unlabelled heparin (Figure 3.2b).

At this stage it was important to determine whether all splenocytes, or a distinct subpopulation were involved in splenic-GAG binding. In order to resolve this issue, splenocytes were reacted with biotinylated-GAGs (Section 2.18) and bound GAGs detected by immunofluorescent flow cytometry (Section 2.19) using an amplification procedure involving fluoresceinated avidin, rabbit anti-avidin antibodies and fluoresceinated sheep anti-rabbit Ig. Typical flow cytometry profiles are depicted in Figure 3.3 and it is evident that, when compared with control binding, the majority of spleen cells bound the splenic-GAGs, although there may be a very small subpopulation of cells (<2%) which exhibit high density binding. Flow cytometry profiles (not shown) revealed splenic-GAG binding at concentrations ranging from 0.8-100 μg/mL, data consistent with the binding assay depicted in Figure 3.1a.

3.2.3 Polysaccharide Specificity of Splenic-GAG Binding

A range of different polysaccharides were tested for their ability to inhibit the binding of ^{125}I-splenic GAGs to spleen cells (Section 2.16). The inhibitory activity of these different polysaccharides and their relevant structural features are presented in Table 3.2. Splenic-GAG binding was almost completely inhibited (approx. 80%) by heparin, pentosan sulfate, fucoidan, dextran sulfate, λ- and ι-carrageenan and partially inhibited (approx. 50%) by κ-carrageenan. Hyaluronic acid, chondroitin-4- and -6-sulfate, dermatan sulfate, keratan sulfate and heparan sulfate (bovine kidney) had little or no effect on GAG binding.



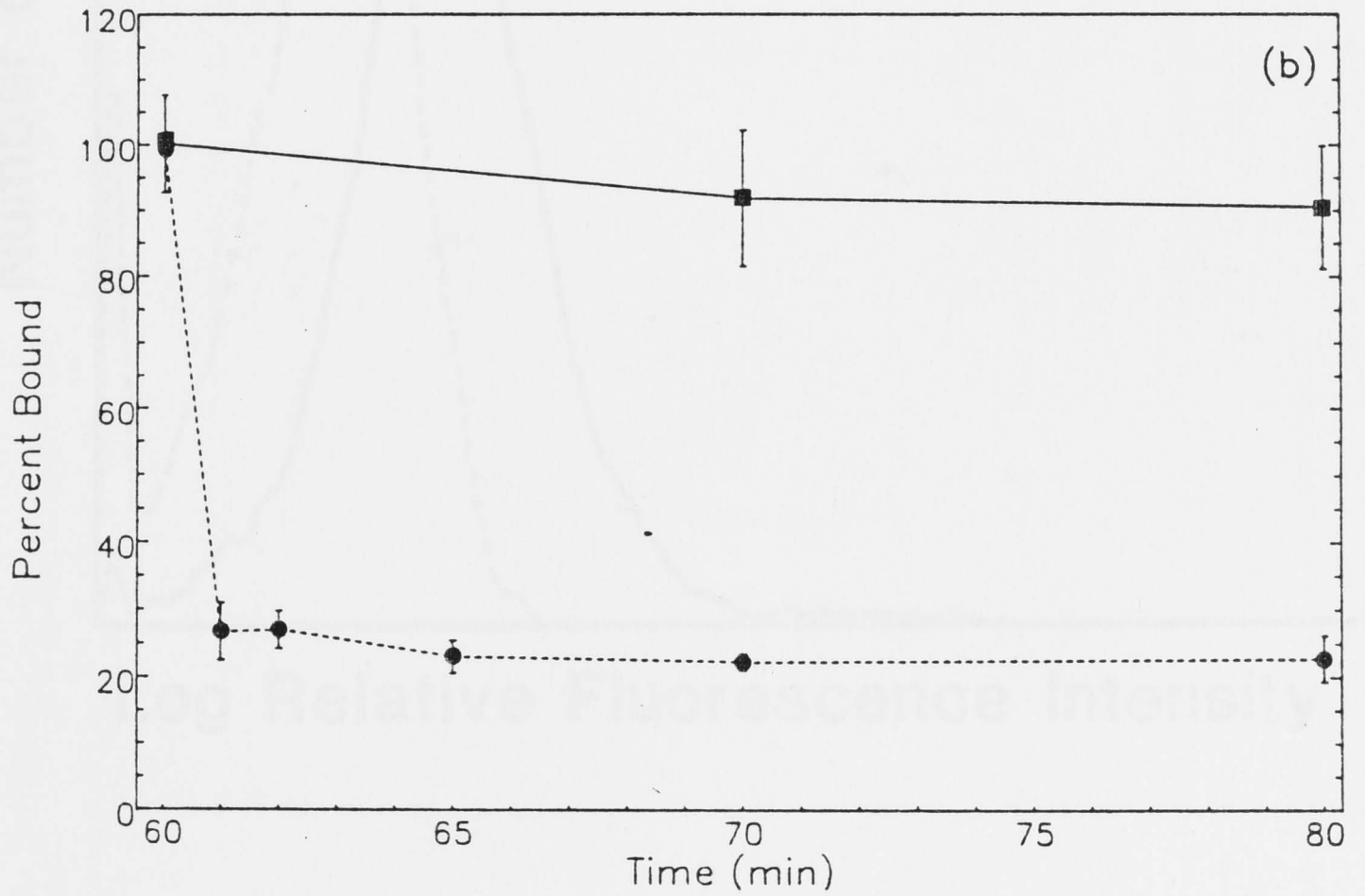


Figure 3.2 Analysis of Binding of Radiolabelled Murine Splenic-GAGs to Splenocytes.

(a) Time course of binding of ^{125}I-splenic GAGs to splenocytes. Data was corrected for nonspecific binding as in Figure 3.1 and each data point is the mean of two replicates. (b) Reversibility of binding of ^{125}I-splenic GAGs to splenocytes in the presence of either no competitor (■) or a 150-fold excess of unlabelled heparin (●). Each data point is the mean of three replicates ± standard error of means. All washing of cells was performed on the cell harvester.

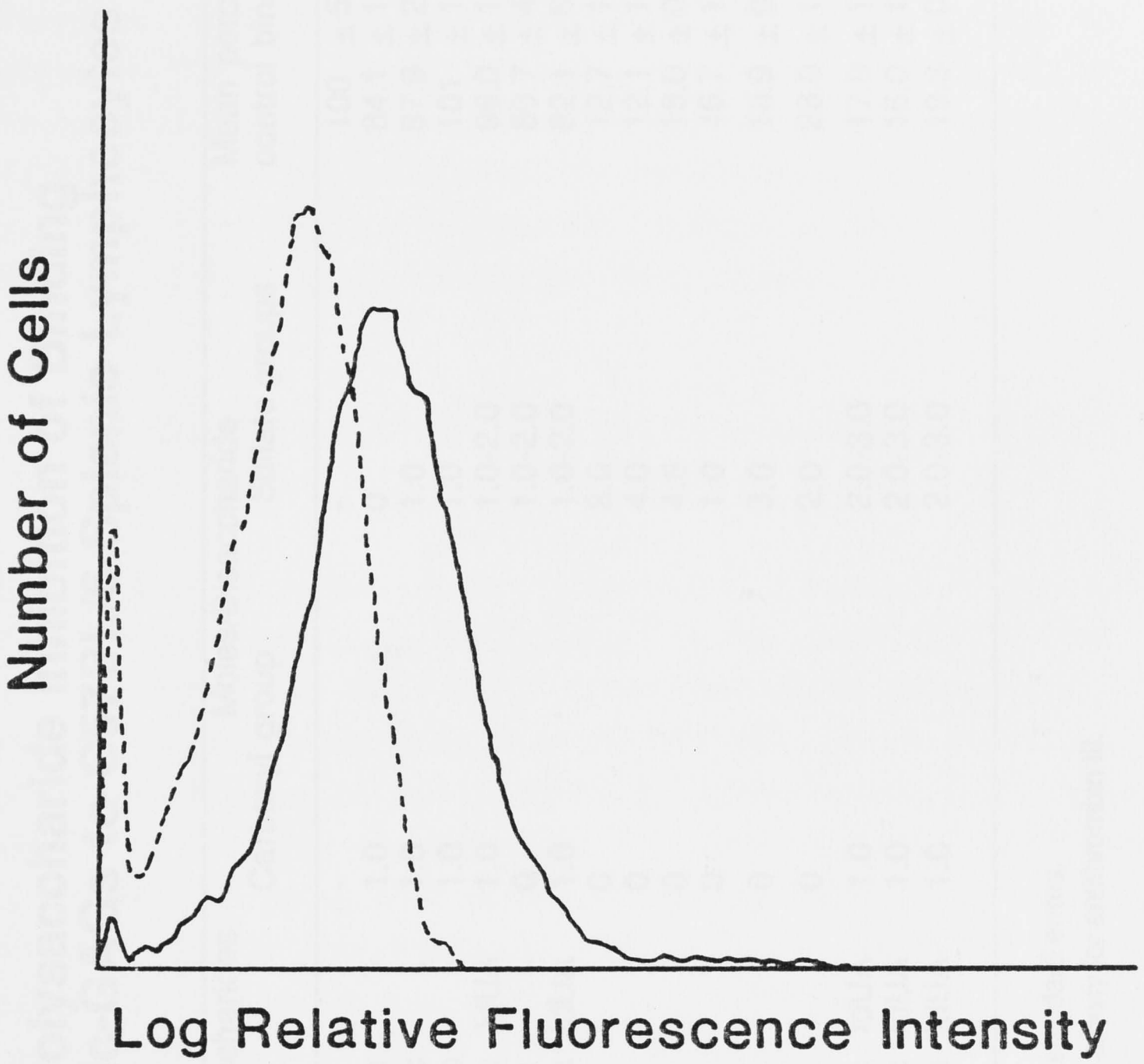


Figure 3.3 Ability of Biotinylated Splenic-GAGs to Bind to Murine Splenocytes as Quantified by Flow Microfluorometry.

Biotinylated splenic-GAGs at 100 μg/mL (——); control profile (- -) representative of cell preincubated with second, third and fourth stage reagents.

Table 3.2 Polysaccharide Inhibition of Binding of C57BL/6 Splenic-GAGs to C57BL/6 Splenic Lymphocytes

Polysaccharide	Major monosaccharides			Moles/Disaccharide		Mean percentage control binding
				Carboxyl group	Sulfate groups	
Nil	-			-	-	100 ± 5.4
Hyaluronic acid	GlcUA	GlcNAc		1.0	0	84.1 ± 1.9
Chondroitin-4-sulfate	GlcUA	GalNAc		1.0	1.0	97.9 ± 2.2
Chondroitin-6-sulfate	GlcUA	GalNAc		1.0	1.0	101 ± 1.0
Dermatan sulfate	GlcUA	GalNAc	IdUA	1.0	1.0-2.0	96.0 ± 1.5
Keratan sulfate	Gal	GlcNAc		0	1.0-2.0	89.7 ± 4.7
Heparan sulfate	GlcUA	GlcNAc	IdUA	1.0	1.0-2.0	82.1 ± 6.1
Fucoidan	L-Fuc			0	2.0	12.7 ± 1.0
Pentosan sulfate	D-Xyl			0	4.0	12.1 ± 1.0
Dextran sulfate	D-Glc			0	4.6	18.0 ± 0.8
κ-carrageenan	D-Gal			0	1.0	46.7 ± 1.9
λ-carrageenan	D-Gal			0	3.0	18.9 ± 0.5
ι-carrageenan	D-Gal			0	2.0	28.0 ± 1.0
Heparin	GlcUA	GlcNAc	IdUA	1.0	2.0-3.0	17.9 ± 1.3
Heparin, high affinity[a]	GlcUA	GlcNAc	IdUA	1.0	2.0-3.0	16.0 ± 1.4
Heparin, low affinity[a]	GlcUA	GlcNAc	IdUA	1.0	2.0-3.0	19.3 ± 0.9

Each percentage is the mean of three data points ± standard errors.

[a] Heparin separated into fractions with high and low affinity for antithrombin III.

Figure 3.4 depicts the ability of hyaluronic acid, chondroitin-4-sulfate, κ-carrageenan and heparin at a range of concentrations (1.6-200 μg/mL) to inhibit splenic-GAG binding. Heparin was a potent inhibitor of GAG binding, inhibiting binding by 85-90% at high concentrations. Furthermore, heparin fractions with either high or low affinity for antithrombin III were equally effective at inhibiting GAG binding. In contrast, hyaluronic acid and chondroitin-4-sulfate had little effect on GAG binding even at the highest concentrations tested whereas κ-carrageenan exhibited an intermediate inhibition profile, partially inhibiting (50-60%) GAG binding to murine spleen cells.

3.2.4 Characterisation of Splenic-GAGs Which Bind to Splenocytes

Chondroitinase digestion of the splenic-GAG preparation demonstrated that the majority of the GAGs belonged to the chondroitin sulfate family (Section 3.2.1; Table 3.1). It was important, therefore, to determine whether chondroitinase ABC and AC digestion had any effect on the binding of splenic-GAGs to murine splenocytes. It was found (Table 3.1) that chondroitinase AC digestion had no effect on GAG binding (i.e. 96 $\pm$ 3.7% of control binding following enzyme treatment) whereas chondroitinase ABC digestion actually resulted in a significant increase in binding (i.e. 130 $\pm$ 4.3% of control binding following digestion). Similarly RNase and DNase digestion of the GAGs had no effect on binding (data not shown). In contrast, nitrous acid treatment resulted in a substantial reduction in GAG binding (i.e.. 56 $\pm$ 3.7% of control binding following treatment). These data indicate that a subpopulation of splenic-GAGs interacts with murine spleen cells and the relevant GAGs were not chondroitin sulfates but probably belong to the heparan sulfate/heparin family.

To further characterise the chondroitinase ABC resistant GAGs the radiolabelled splenic-GAG preparation was chondroitinase ABC digested prior to application and fractionation on a DEAE-Sepharose

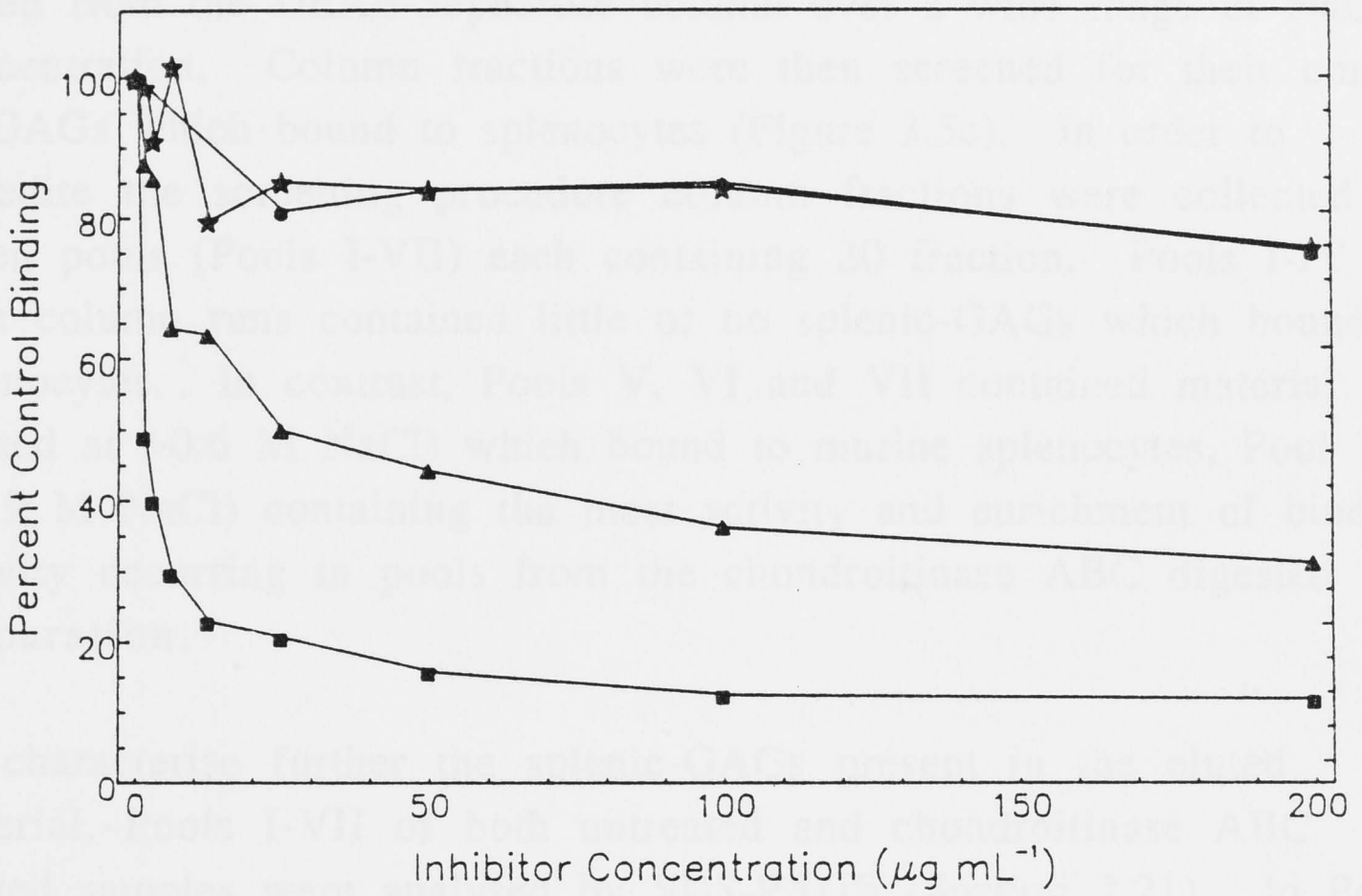


Figure 3.4 Inhibition of Binding of ^{125}I-Splenic GAGs to Splenocytes using Unlabelled GAGs.

Unlabelled GAGs used were hyaluronic acid (★), chondroitin-4-sulfate (●), κ-carrageenan (▲) and heparin (■). Results are expressed as the percentage of splenic-GAGs binding in the absence of inhibitor. Washing of cells was performed on the cell harvester. Each data point is the mean of two replicates.

column (Section 2.17). The fractionation profile was compared with that obtained for undigested splenic-GAGs (Figure 3.5). With the undigested GAG preparation the bulk of the radioactivity was eluted at >0.6 M NaCl. As was previously observed a large proportion of the splenic-GAG preparation was destroyed by chondroitinase ABC digestion (Figure 3.5b). However, the remaining radioactivity was eluted from the DEAE-Sepharose column over a wide range of NaCl concentration. Column fractions were then screened for their content of GAGs which bound to splenocytes (Figure 3.5c). In order to expedite the screening procedure column fractions were collected into seven pools (Pools I-VII) each containing 20 fraction. Pools I-IV from both column runs contained little or no splenic-GAGs which bound to splenocytes. In contrast, Pools V, VI and VII contained material (eluted at >0.6 M NaCl) which bound to murine splenocytes, Pool VII (>0.9 M NaCl) containing the most activity and enrichment of binding activity occurring in pools from the chondroitinase ABC digested preparation.

To characterise further the splenic-GAGs present in the eluted material, Pools I-VII of both untreated and chondroitinase ABC treated samples were analysed by SDS-PAGE (Section 2.21). In Pools V-VII from both untreated and chondroitinase treated samples, radiolabelled material was detected throughout the gel, a pattern characteristic of the polydispersed nature of GAGs (Figure 3.6a and b). Nevertheless, chondroitinase digestion tended to destroy the high MW material (>200 kDa) and enriched for low MW GAG sidechains close to the dye front, particularly in Pools VI and VII. Subsequent nitrous acid treatment (Figure 3.6c) resulted in total elimination of the chondroitinase resistant material in Pools IV-VII indicating that the residual GAGs were heparan sulfate/heparin in nature. Additional SDS-PAGE runs on 15% gels revealed that the mean MW of chondroitinase ABC resistant GAGs in Pools VI and VII was approximately 50 kDa (range 10-50 kDa) (data not shown). It should be emphasised that this estimate is somewhat arbitrary due to the use

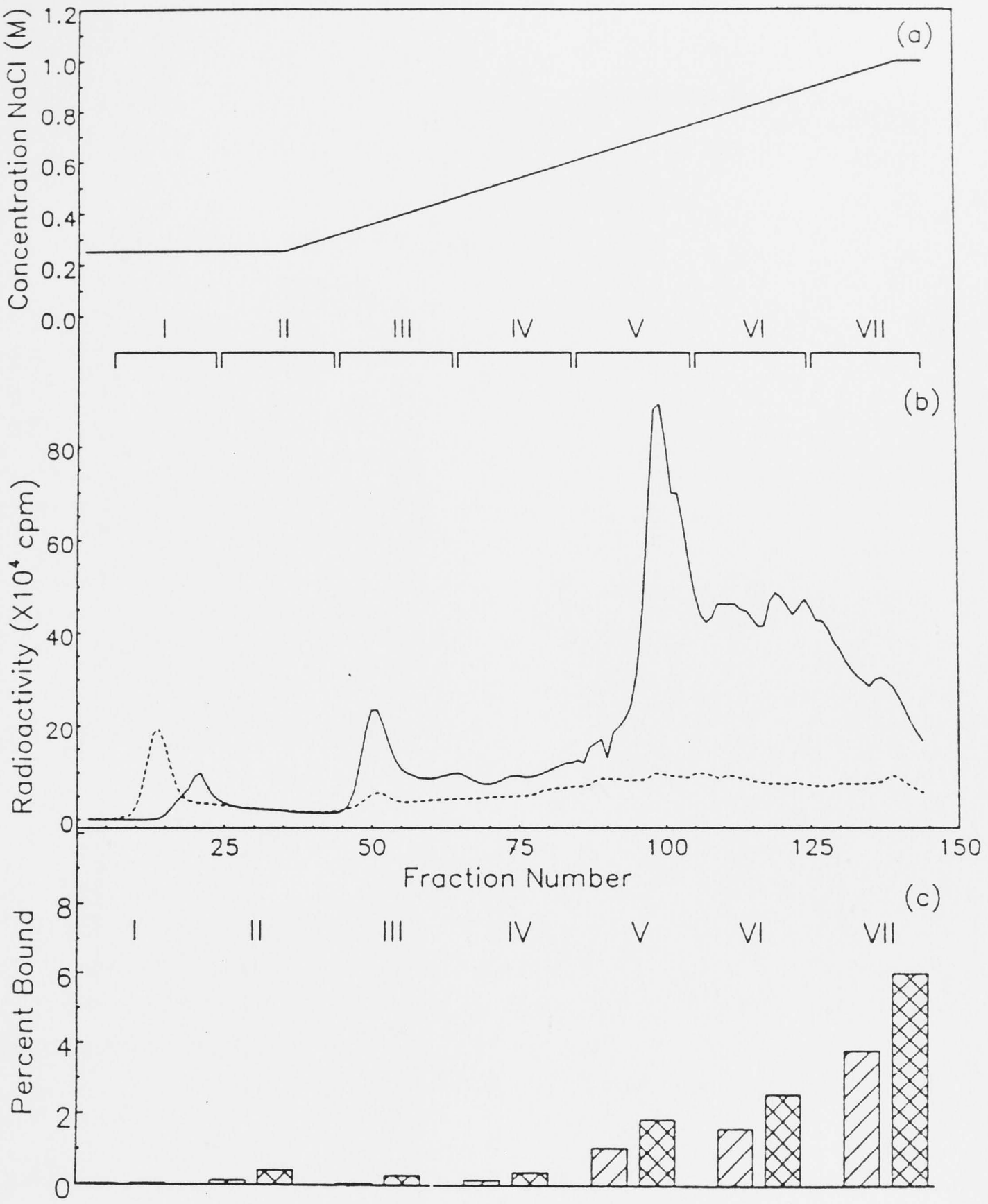
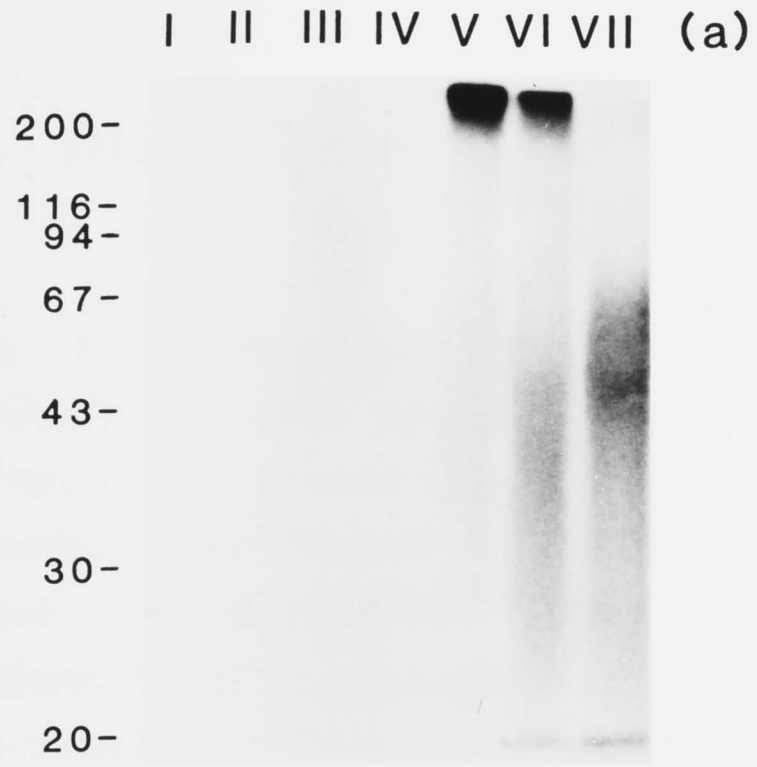


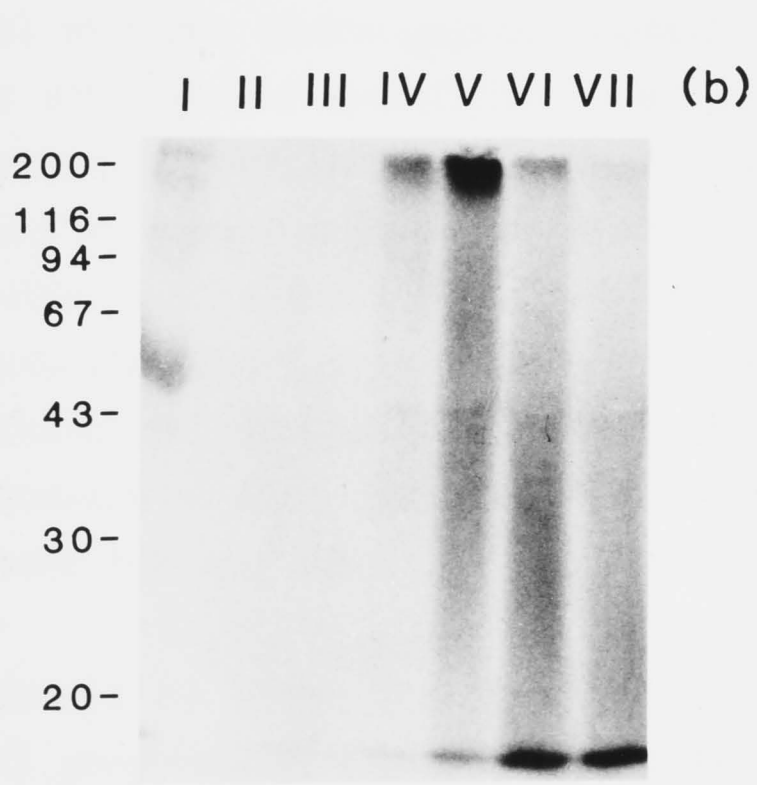
Figure 3.5 Ion Exchange Chromatography of an ^{125}I-Splenic GAG Preparation.

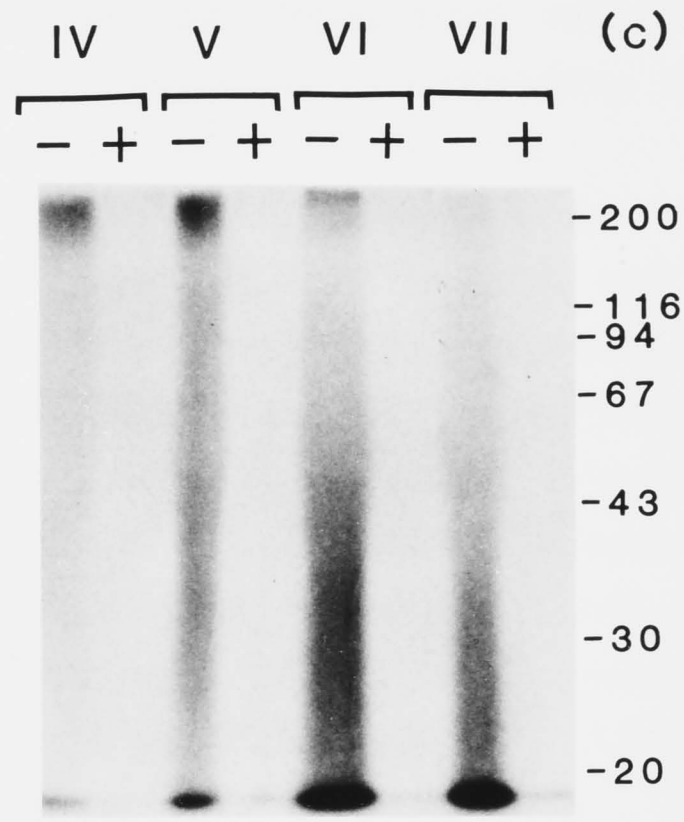
An ^{125}I-Splenic GAG preparation either untreated or digested with chondroitinase ABC, was applied to a DEAE Sepharose CL6B column and eluted with a 0.25-1.0 M NaCl gradient in 10 mM Tris-HCl buffer, pH 7.0. (a) Salt gradient generated during column run. (b) Radioactivity detected in different column fractions with untreated (——) or chondroitinase ABC digested (- -) splenic-GAGs. Fractions were collected into Pools I-VII as indicated. (c) Ability of radioactive material in Pools I-VII to bind to murine splenocytes. The percent bound for untreated (▨) and chondroitinase ABC treated (▩) splenic-GAGs is given. Washing of cells was performed by centrifugation.

Figure 3.6 SDS-PAGE Analysis of ^{125}I-Splenic GAG Pools Isolated by Ion Exchange Chromatography.

(a) Pools I-VII from untreated splenic-GAGs. Exposure time 24 h. (b) Pools I-VII from chondroitinase ABC treated splenic-GAGs. Exposure time, 7 days. (c) Pools IV-VII from chondroitinase ABC treated splenic-GAG which were either nitrous acid digested (+) or untreated (-). Exposure time, 7 days. The position of molecular weight markers is indicated in kDa.


I II III IV V VI VII (a)
200-
116-
94-
67-
43-
30-
20-


I II III IV V VI VII (b)
200-
116-
94-
67-
43-
30-
20-


IV V VI VII (c)
- + - + - + - +
-200
-116
-94
-67
-43
-30
-20

of protein rather than defined polysaccharide standards when determining the GAG molecular weights.

3.3 DISCUSSION

This chapter describes attempts to isolate and characterise sulfated-GAGs from murine spleen which could be involved in controlling the migration and positioning of murine splenic lymphocytes. A procedure was devised for isolating sulfated-GAGs from murine spleen in good yield and high purity (Figure 2.1; Section 2.13) and the resultant GAG preparation was used in subsequent binding studies. It was found that isolated splenic-GAGs bound to murine splenocytes in a saturable (Figure 3.1a), rapid (Figure 3.2a) and reversible (Figure 3.2b) manner, characteristics consistent with a cell surface receptor of high affinity and specificity. Further analysis of the splenic-GAG preparation revealed that a small subpopulation of the GAG molecules bound to spleen cells. The reactive GAGs were chondroitinase ABC resistant and nitrous acid sensitive, properties consistent with polysaccharides belonging to the heparan sulfate/heparin family. Furthermore, immunofluorescent flow cytometry studies demonstrated that the majority of murine splenic lymphocytes express binding sites for the relevant splenic-GAGs.

It should be noted that nitrous acid treatment of the 'crude' splenic-GAG preparation reduced binding to spleen cells by only 44% (Table 3.1). However, subsequent studies revealed that GAGs present in DEAE column fractions highly enriched for binding activity were entirely nitrous acid sensitive as judged by SDS-PAGE analysis (Figure 3.6). This discrepancy may be due to nitrous acid producing small fragments (for example tetra/pentasaccharides) which are not detected by SDS-PAGE but still retain some binding activity.

Based on the data presented an estimate can be made of the binding affinity of the lymphocyte receptors for splenic-GAGs. Since probably only 15% of the splenic-GAG preparation was involved in binding and assuming a mean MW of the GAGs of 50 kDa, the binding sites on spleen cells have an apparent affinity constant of 5×10^{-8} M. This affinity is 50-fold higher than the affinity of the interaction between heparin and antithrombin III (Lindahl and Höök, 1978).

Some interesting features of the splenocyte/splenic-GAG interaction were revealed by the binding inhibition studies (Table 3.2, Figure 3.4). Firstly, out of 13 polysaccharides tested, only seven (heparin, fucoidan, pentosan sulfate, dextran sulfate, κ-, λ- and ι-carrageenan) inhibited binding (Table 3.2). Charge density alone cannot account for the binding inhibition observed as chondroitin-4- and -6-sulfate and dermatan sulfate have a higher charge density than κ-carrageenan and yet only the latter polysaccharide inhibited binding. Similarly ι-carrageenan and fucoidan inhibited binding, whilst the chondroitin sulfates (of similar charge density) did not. Secondly, minor changes in the sulfation pattern of the polysaccharides had substantial effects on inhibitory activity as κ- and ι-carrageenan, which differ only in the number of sulfates per disaccharide (one and two, respectively), showed significant differences in their ability to inhibit GAG binding. Thirdly, the observation that κ-carrageenan only partially inhibited splenic-GAG binding suggests that splenocytes may contain more than one type of binding site on their cell surface, one site not reacting with κ-carrageenan and the other site reacting with all seven inhibitory polysaccharides. Similarly, diversity of binding sites for SPS has been detected on murine thymocytes (Parish *et al.*, 1988). Fourthly, heparin fractions with either high or low affinity for antithrombin III exhibited similar inhibitory activity indicating that the antithrombin III binding site on heparin plays little or no role in splenocyte binding.

Although the splenic-GAGs which bind to lymphocytes appear to belong to the heparan sulfate/heparin family it is interesting to note

that heparan sulfate isolated from bovine kidney did not inhibit splenic-GAG binding. This observation is not particularly surprising as heparan sulfates exhibit great structural diversity, earlier studies suggesting that theoretically 10^{36} types of heparan sulfate could occur in animal tissues (Dietrich *et al.*, 1983). Presumably therefore, bovine kidney heparan sulfate, which is less sulfated than heparin, does not contain the correct positioning of sulfates for binding to occur. In this context, cell surface receptors which can distinguish subtle changes in the structure of SPS have been detected on a range of cell types such as lymphocytes (Parish *et al.*, 1988; Parish and Snowden, 1985), macrophages (Chong and Parish, 1986), endothelial cells (Glabe *et al.*, 1983b) and neurones (Vidovic *et al.*, 1986).

3.4 SUMMARY

Previous studies have shown that lymphocytes carry cell surface receptors for SPS and SPS recognition may play a role in lymphocyte migration and positioning *in vivo*. This chapter describes attempts to isolate and characterise the endogenous GAGs of murine spleen and determine whether splenic lymphocytes carry cell surface receptors for these GAGs.

A procedure was devised for isolating GAGs from murine spleen in good yield and high purity and the GAG preparation was then radiolabelled for subsequent binding studies. It was found that the splenic-GAGs bound to murine splenocytes in a saturable, rapid and reversible manner with only a small subpopulation of the splenic-GAG preparation being involved in binding. This reactive species was chondroitinase ABC resistant and nitrous acid sensitive, indicative of a heparan sulfate/heparin-like molecule. Furthermore, using immunofluorescent flow cytometry studies it was demonstrated that the majority of spleen cells have receptors for these GAGs.

Subsequent ion exchange fractionation and SDS-PAGE analysis of chondroitinase ABC resistant GAGs confirmed that the splenic-GAG recognised by splenocytes was a heparan sulfate/heparin molecule of approx. 50 kDa with a binding affinity to splenocytes of approx. 5 x 10^{-8} M.

Additional binding inhibition studies indicated two possible binding sites for splenic-GAGs on the splenocyte surface, one being fully inhibited by a range of SPS such as heparin (both coagulant and anticoagulant forms), pentosan sulfate, fucoidan, dextran sulfate, λ- and ι-carrageenan, and the second being partially inhibited by κ-carrageenan.

CHAPTER 4 : CHARACTERISATION OF GLYCOSAMINOGLYCAN RECEPTORS ON LYMPHOCYTES

4.1 INTRODUCTION

A vast amount of literature has been published recently on the identification and characterisation of cell surface antigens on lymphocytes particularly those involved in cellular adhesion events and lymphocyte recognition processes (Table 1.1, 1.2). Some of these molecules, such as MEL-14 and CD44, have been implicated in lymphocyte migration and in particular, appear to play a role in the interaction of lymphocytes with HEV (Section 1.6.1). However, despite such findings, the endogenous ligands for many of these molecules are unknown. In this context, a large body of data suggests that carbohydrate recognition systems may play a role in many forms of cell adhesion and lymphocyte migration (Section 1.7). Evidence for this includes the modification of lymphocyte migration by carbohydrate modifiers such as glycosidases, periodate and swainsonine (Section 1.7.1); by lectins (Section 1.7.2) and polysaccharides (Section 1.7.3).

Following the demonstration that splenocytes interact with endogenous splenic-GAGs in a highly avid and specific manner (Chapter 3), this chapter describes attempts to identify and characterise defined molecules on the lymphocyte surface which interact with these GAGs. Of particular interest was whether well characterised cell surface molecules, particularly those implicated in lymphocyte migration, bind GAGs.

4.2 RESULTS

4.2.1 IDENTIFICATION OF GAG-BINDING PROTEINS ON THE SURFACE OF LYMPHOCYTES

Initially three approaches were used to detect receptors for GAGs on the surface of lymphocytes. Firstly, binding studies such as those described in Chapter 3, were performed where ^{125}I-labelled GAGs were bound to murine lymphocytes and the binding data assessed. This approach gave a gross indication of whether GAG-binding molecules were present on the lymphocyte surface. Secondly, GAG-coupled erythrocytes were examined for their ability to form rosettes with murine lymphocytes. As with the binding studies using radiolabelled GAGs, this approach only gave a gross assessment of the presence or absence of cell surface receptors for GAGs. Nevertheless, rosetting assays have the advantage that, due to multipoint binding of erythrocytes, they are much more sensitive than binding assays using radiolabelled GAGs. Furthermore, with a rosetting procedure the percentage of lymphocytes carrying receptors for a particular GAG can be assessed. Thirdly, the molecular nature of cell surface receptors for GAGs was determined by binding ^{125}I-cell surface labelled lysates of lymphocytes to immobilised GAGs and characterising bound molecules by SDS-PAGE. This approach could be used to identify specific cell surface receptors for GAGs on splenocytes and thymocytes.

A range of polysaccharides, namely, hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparin and heparan sulfate were radiolabelled with ^{125}I (Section 2.15) and their binding to splenocytes assessed (Section 2.16). The data presented in Figure 4.1 depicts the binding of ^{125}I-labelled heparin and ^{125}I-labelled chondroitin-4-sulfate to splenocytes and was corrected for non-specific binding by the presence of a 50-fold excess of unlabelled heparin or chondroitin-4-sulfate, respectively.

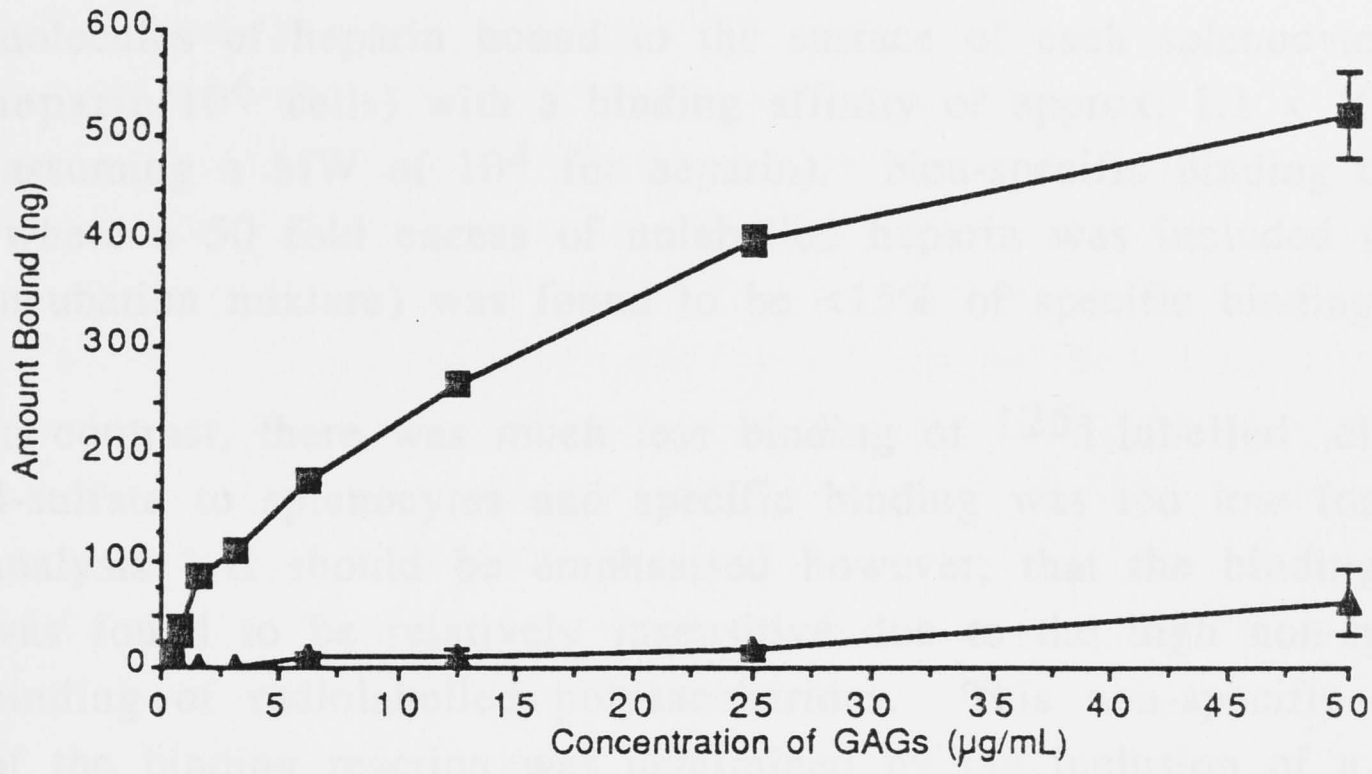


Figure 4.1 Analysis of Binding of ^{125}I Labelled GAGs to Murine Splenocytes

Concentration dependence of binding of ^{125}I labelled chondroitin-4-sulfate (▲) and heparin (■) to murine splenocytes. The data shown have been corrected for nonspecific binding by subtracting the amount of radioactivity bound in the presence of a 50-fold excess of unlabelled GAG. Each data point is the mean of three replicates ± standard error.

The binding of heparin to splenocytes shows a typical binding curve indicative of saturable binding and is similar to that observed for the binding of endogenous splenic-GAGs to splenocytes (Figure 3.1a). A Scatchard plot of data (not shown) indicated that the line approached linearity (correlation coefficient 0.81) with approx. 9.1×10^6 molecules of heparin bound to the surface of each splenocyte (151 ng heparin/10^6 cells) with a binding affinity of approx. 1.1×10^{-6} M (assuming a MW of 10^4 for heparin). Non-specific binding of heparin (where a 50 fold excess of unlabelled heparin was included in the incubation mixture) was found to be <15% of specific binding.

In contrast, there was much less binding of ^{125}I-labelled chondroitin-4-sulfate to splenocytes and specific binding was too low for Scatchard analysis. It should be emphasised however, that the binding assay was found to be relatively insensitive due to the high non-specific binding of radiolabelled polysaccharides. This non-specific component of the binding reaction was determined by the inclusion of a 50 fold excess of unlabelled polysaccharide in the incubation mixture. For example, in the case of chondroitin-4-sulfate it was calculated that maximum non-specific binding at 50 μg/mL was 3.4×10^5 molecules/cell and similar non-specific binding was observed with hyaluronic acid, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate and heparan sulfate. This implied that to calculate accurately the binding affinities of GAGs to lymphocytes, under saturating conditions much greater than 3.4×10^5 molecules of ^{125}I-labelled GAGs would need to be bound to each cell. Obviously in the case of heparin and endogenous splenic-GAGs, where large numbers of molecules bound to the splenocyte surface (4.2×10^6 and 9.1×10^6 molecules respectively), this assay is feasible. However, when $<3.4 \times 10^5$ polysaccharide molecules bound to the surface of each cell another method needed to be employed to detect GAG receptors. Thus in order to detect relatively low numbers of GAG receptors on lymphocytes the rosetting approach was used.

The rosetting of murine lymphocytes to PS-coupled SRBC was carried out as described in Section 2.7. Results of this assay presented in Table 4.1 indicate that between 20% and 90% of lymphocytes express receptors for GAGs. Both splenocytes and thymocytes carried receptors for hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate and heparin although differences were seen between the percent rosetting for thymocytes and splenocytes with different GAGs. For example, only 21% of thymocytes expressed receptors for hyaluronic acid whereas 62% of splenocytes expressed receptors for this non-sulfated GAG (Table 4.1). In contrast, a high proportion (82-90%) of both splenocytes and thymocytes rosetted with heparin-coupled erythrocytes. Thus, the rosetting studies indicate that lymphocytes express receptors for all classes of GAGs, although binding studies with radiolabelled heparin imply that there are substantially more binding sites on the lymphocyte surface for heparin than for the other GAGs.

Table 4.1 Rosetting of Lymphocytes from Thymus and Spleen with GAG-Coupled Sheep Erythrocytes

GAG	Rosettes (%)	
	Spleen	Thymus
Hyaluronic acid	62.3 ± 2.9	21.0 ± 1.5
Chondroitin-4-sulfate	62.7 ± 1.9	54.3 ± 3.2
Chondroitin-6-sulfate	72.3 ± 4.2	64.3 ± 1.2
Heparin	82.0 ± 1.7	90.3 ± 0.3

Each value is the mean of three replicates ± standard errors.

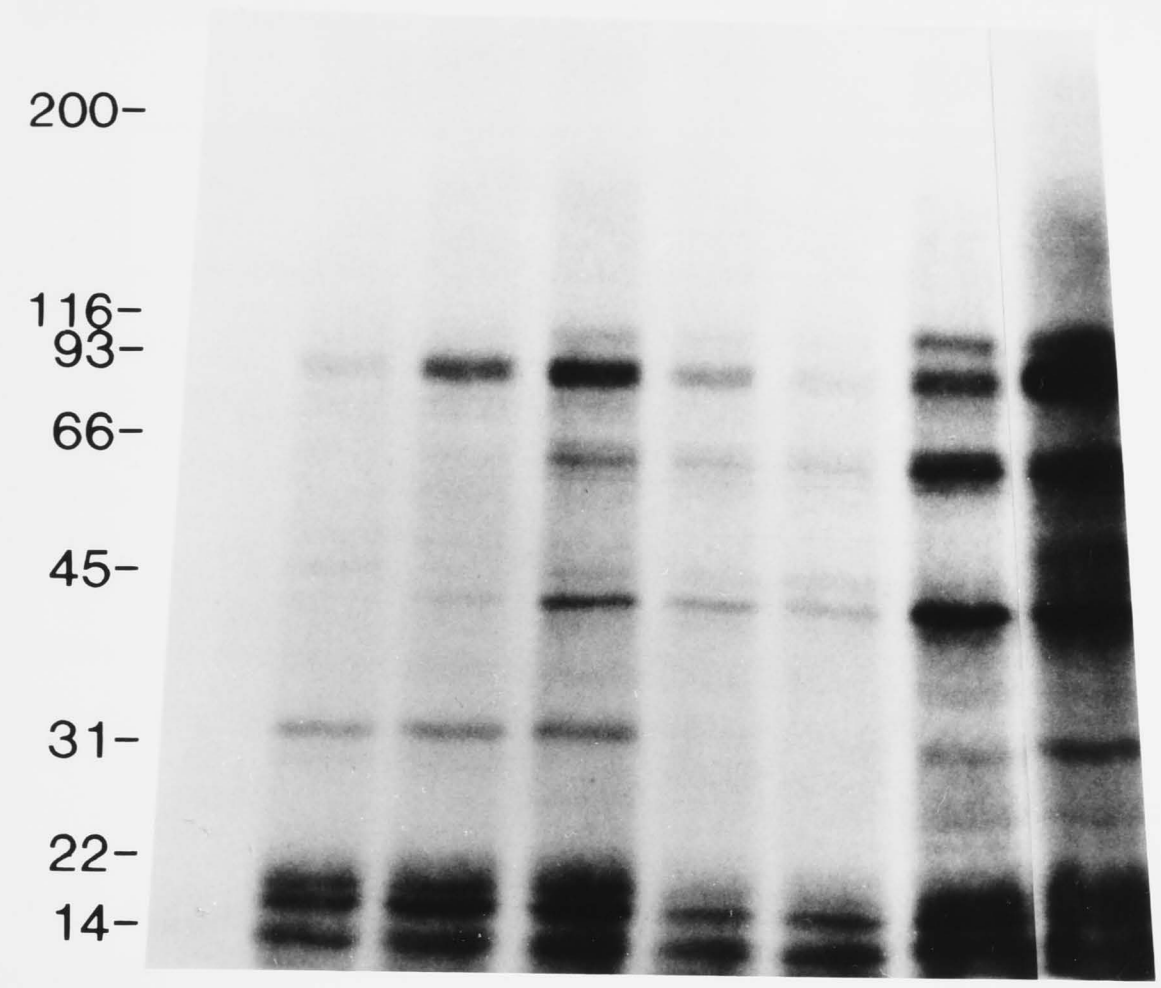
In an attempt to characterise GAG binding molecules on the surface of lymphocytes ^{125}I-labelled cell surface molecules were bound to a variety of GAGs (hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, endogenous splenic-GAGs and heparin) immobilised on CMC fibres (Section 2.9, 2.10) and the bound molecules characterised by SDS-PAGE. The results of such an analysis are shown in Figure 4.2 and indicate that a number of molecules on the surface of splenocytes and thymocytes bind to GAGs. Murine splenocytes were seen to express at least 10 distinct GAG-binding molecules with a wide range of molecular masses (i.e., approx. 10-20, 33, 40, 60, 90 and 100 kDa). However, each GAG bound to a unique profile of cell surface molecules (Figure 4.2; Table 4.2). For example, chondroitin-4-sulfate bound predominantly to molecules with molecular masses of 10-20, 33 and 90 kDa whereas chondroitin-6-sulfate bound additional molecules of 40 and 60 kDa. The reactivity of particular molecules also varied between GAGs, for example a 90 kDa protein on splenocytes reacting strongly with chondroitin-4-sulfate and chondroitin-6-sulfate, moderately with dermatan sulfate and weakly with hyaluronic acid and heparan sulfate (Table 4.2). Furthermore, differences were seen between the splenocyte and thymocyte receptor profiles, the latter expressing a similar pattern of cell surface molecules which bound to each GAG but with additional molecules at 190 and 250 kDa when compared with spleen. The very prominent 90 kDa GAG-binding molecule which was detected on the thymocyte and splenocyte surface will be characterised in detail in Chapter 5. However, the binding data presented in Figure 4.2 suggests that the 90 kDa GAG-binding protein differs in its GAG specificity between thymus and spleen.

Finally, the molecules which bound to endogenous splenic-GAGs include all the major GAG-binding proteins, namely molecules with molecular masses of approx. 10-20, 33, 40, 60, 90 and 100 kDa on splenocytes and additional molecules of 190 and 250 kDa on thymocytes.

Figure 4.2 Analysis of ^{125}I-Labelled Splenocyte Cell Surface Molecules Which Bind to Immobilised GAGs

Bound molecules were run on a 8-18% gradient SDS-PAGE gel and gels were autoradiographed for two days except in the case of heparin-binding molecules where gels were exposed for one day. Molecular weight markers are indicated in kDa.
HA=hyaluronic acid, Ch4S=chondroitin-4-sulfate, Ch6S=chondroitin-6-sulfate, DermS=dermatan sulfate, HepS=heparan sulfate, GAG=endogenous splenic-GAGs, Hep=heparin.

Splenocyte



Thymocyte

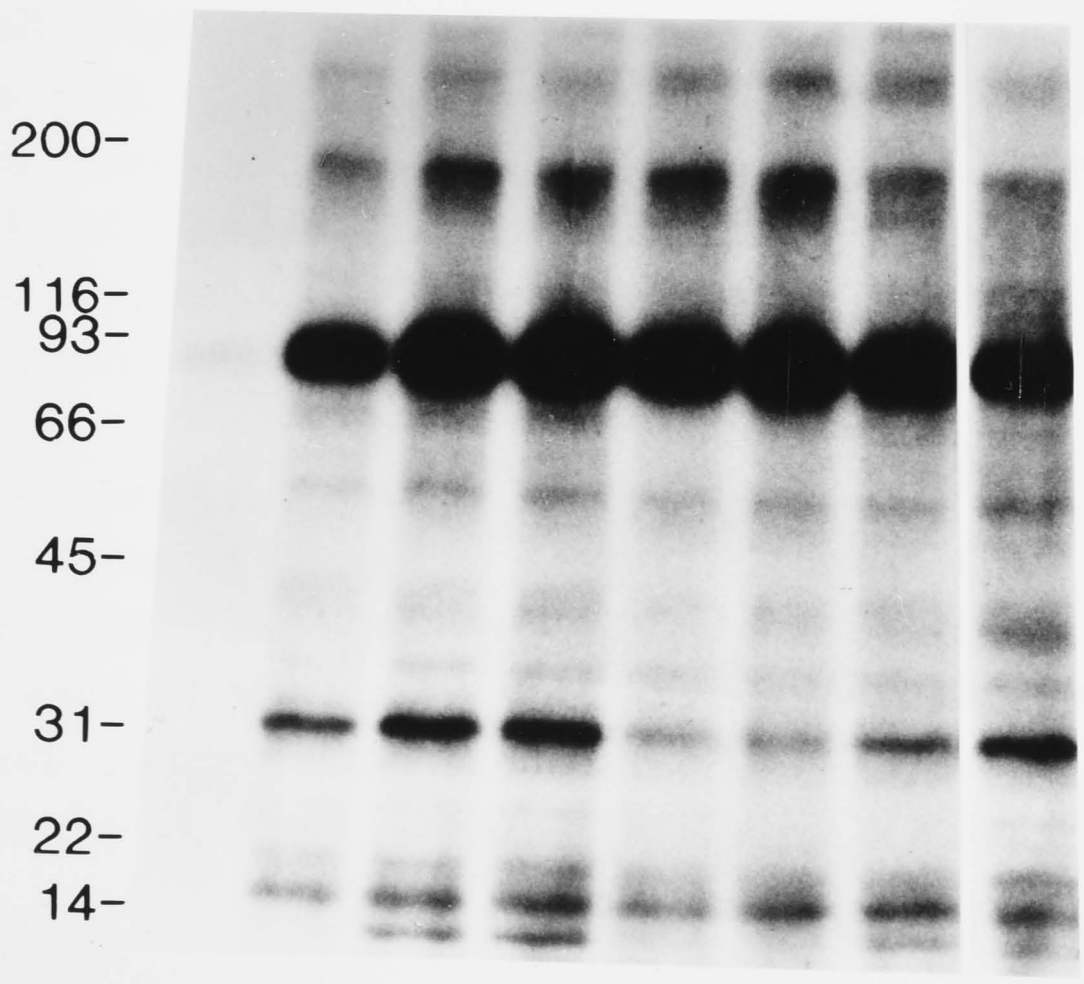


Table 4.2 Summary of GAG-Binding Molecules on the Surface of Murine Splenocytes

GAG	GAG-binding molecule (kDa)					
	10-20	33	40	60	90	100
Hyaluronic acid	+	+	-	-	tr	-
Chondroitin-4-sulfate	+	+	-	-	++	-
Chondroitin-6-sulfate	+	+	+	+	++	+
Dermatan sulfate	+	-	+	tr	+	-
Heparan sulfate	+	-	+	tr	tr	-
Splenic-GAGs	+	+	++	++	++	+
Heparin	+	+	++	++	++	+

- = no detectable binding, tr = trace binding, + = binding, ++ = strong binding

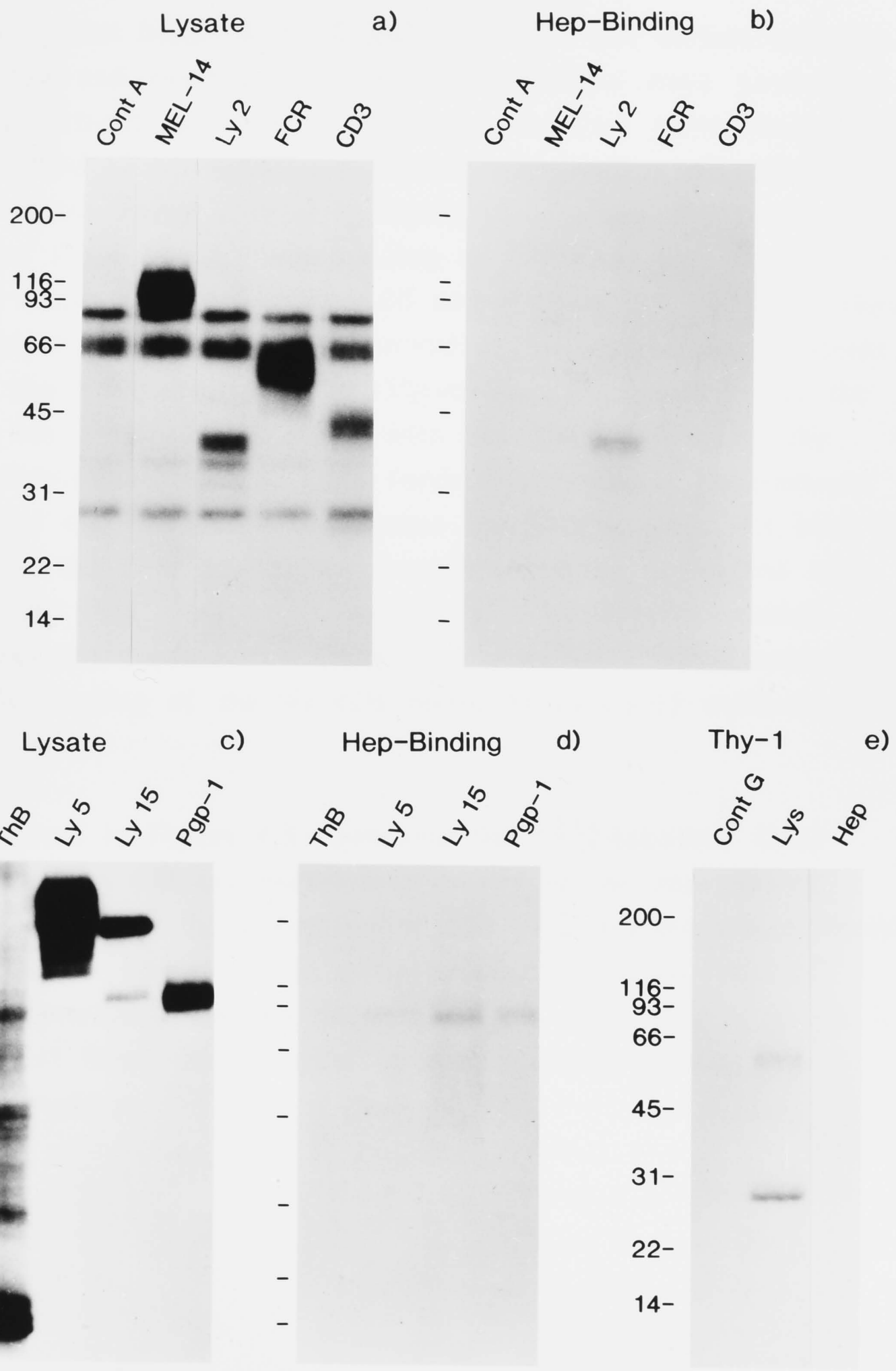
4.2.2 Characterisation of GAG Receptors on Splenocytes

To determine if any of the cell surface molecules which bound to GAGs corresponded to known lymphocyte cell surface antigens and especially those involved in cell adhesion events, immunoprecipitation studies were performed on heparin-binding molecules isolated from ^{125}I-labelled splenocyte lysates (Section 2.20). Heparin-binding molecules were obtained by binding ^{125}I-labelled splenocyte lysates to heparin immobilised on CMC fibres and eluting bound molecules with 2 M NaCl (Section 2.9, 2.10). Preparations of heparin-binding molecules were used as they contained all GAG-binding molecules detected (Table 4.2; Figure 4.2).

The mAbs tested are listed in Table 2.1 and some typical immunoprecipitation results are depicted in Figure 4.3. The MEL-14 , Ly-2, FcR, CD3, ThB, Ly-5, Ly-15, Pgp-1 and Thy-1 cell surface antigens were all immunoprecipitated from ^{125}I-labelled splenocyte lysates with corresponding molecular masses of approx. 95 (MEL-14), 36 and 38 (Ly-2), 48-68 (FcR), 41 (CD3), 12-20 (ThB), 190-210 (Ly-5),

Figure 4.3 Ability of Different Splenocyte Cell Surface Antigens to Bind to Immobilised Heparin

Each mAb was used to monitor the presence of radiolabelled antigen in either an unfractionated splenocyte lysate or ^{125}I-labelled splenocyte molecules eluted from heparin-CMC fibres. Monoclonal antibodies were bound to sheep anti-rat coupled (a and b), protein A coupled (c and d) or protein G coupled (e) Sepharose 4B. Exposure times varied from 7, 22, 1, 4 and 6 days for a, b, c, d and e respectively. Molecular weight markers are indicated in kDa.


Lysate
a)
Hep-Binding
b)
Cont A
MEL-14
Ly 2
FCR
CD3
200-
116-
93-
66-
45-
31-
22-
14-
Lysate
c)
Hep-Binding
d)
Thy-1
e)
ThB
Ly 5
Ly 15
Pgp-1
Cont G
Lys
Hep

100 and 182 (Ly-15), 96 (Pgp-1) and 27 kDa (Thy-1). These values correspond reasonably well with previously published values.

The mAbs against MEL-14, Ly-2, FcR and CD3 cell surface antigens were rat Igs and therefore, immunoprecipitations were performed with sheep anti-rat Ig beads. Although extensive preincubation steps were performed (Section 2.20) to reduce the binding of radiolabelled mouse Ig to the sheep anti-rat Ig beads, due to residual cross reactivity of these beads, some binding of mouse Ig still occurred, i.e., two heavy chain bands of approx. 66 and 80 kDa and one light chain band of approx. 29 kDa were observed in all immunoprecipitations involving sheep anti-rat Ig beads (Figure 4.3a). Nevertheless, the mouse Ig bands did not interfere with the interpretation of the immunoprecipitation results and, furthermore, these experiments revealed that the surface Ig on murine splenocytes does not bind to heparin. In the case of the immunoprecipitations performed with Protein A and Protein G beads, non-specific binding of labelled material was not a problem. There was however, a small amount of non-specific binding of the 90 kDa molecule to Ly-15 mAb-coupled beads.

Results depicted in Figure 4.3 show that the ^{125}I-labelled GAG-binding molecules did not correspond to any of the cell surface antigens tested, with the exception of the Ly-2 antigen which weakly bound to heparin fibres, i.e. in order to clearly discern the Ly-2 immunoprecipitated from the heparin eluate, the autoradiograph had to be exposed three times longer than the control (lysate) immunoprecipitate.

4.3 DISCUSSION

This chapter describes attempts to isolate and characterise receptors for GAG molecules on the splenocyte and thymocyte surface.

Studies reported in this chapter using radiolabelled GAGs demonstrated that the binding of heparin to splenocytes showed a typical saturable binding curve (Figure 4.1) with a large number of binding sites for heparin (9.1×10^6 heparin molecules bound/cell) estimated to be present on the splenocyte surface. However these binding studies were not sensitive enough to determine the binding characteristics of other GAG molecules to the splenocyte surface. The binding affinity for heparin was calculated to be 1.1×10^{-6} M, (assuming a MW of 10^4 for heparin) which indicates a less avid interaction than that demonstrated previously for endogenous splenic-GAGs to splenocytes (5×10^{-8} M; Section 3.2.2). In this context it should be noted that heparin-like molecules in the splenic-GAG preparation were the major binding species (Section 3.2.4). Thus, it appears that the heparin-like GAGs in murine spleen have unique structural features which result in more avid recognition by murine splenocytes, a property not unexpected for an endogenous ligand.

Both rosetting studies (Table 4.1) and receptor profiles (Figure 4.2) provided more sensitive assays for assessing GAG-binding and these demonstrated that splenocytes and thymocytes have receptors for all GAGs tested. Rosetting studies showed that between 20% and 90% of lymphocytes express receptors for GAGs. Splenocytes were found to possess a unique family of cell surface molecules which reacted with each GAG (hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, heparin and endogenous splenic-GAGs), an observation which was much less evident with thymocytes. For example, a 33 kDa splenocyte molecule did not bind to dermatan sulfate and heparan sulfate but bound all other GAG species. Similarly, a 60 kDa molecule bound to all species of GAGs

except chondroitin-4-sulfate and hyaluronic acid. Furthermore, differences were seen in the intensity of the reaction of certain splenocyte molecules with different GAGs (Figure 4.2; Table 4.2). This indicates that the interaction between particular GAGs and their receptors on the lymphocyte surface is very precise.

An interesting feature of the reactivity profile of GAG receptor molecules is the differences seen between GAG receptors on thymocytes and splenocytes. Thymocytes displayed a similar pattern of cell surface molecules which bound to each GAG when compared to that seen for splenocytes. This would be as expected, for thymocytes constitute a relatively homogeneous population of T cells which have not yet begun the process of migration to the peripheral lymphoid organs (Section 1.2). Splenocytes, on the other hand, are a heterogeneous population of cells including migrating and non-migrating T and B lymphocytes and a small subpopulation of non-lymphoid cells (Section 1.2.2) and so would be expected to express a variety of GAG receptors some of which are possibly involved in lymphocyte migration processes.

Another interesting feature of thymocytes is the presence of GAG-binding molecules of approx. 190 and 250 kDa which reacted with all GAG species tested. These GAG-binding molecules were not detected on the surface of splenocytes. The 190 kDa thymocyte molecule is probably the cell surface antigen T200 or Ly-5, which has been shown previously to bind SPS (Parish *et al.*, 1988a) whereas the identity of the 250 kDa molecule is unknown.

Endogenous splenic-GAGs isolated from murine spleen which bound avidly and specifically to splenocytes (Section 3.2.2, 3.2.3), bound a similar profile of GAG-binding molecules (Figure 4.2) to that observed for heparin and heparan sulfate (although the latter expressed a weaker binding pattern). This is in agreement with previous results (Section 3.2.4) where endogenous splenic-GAGs which bound to murine splenocytes were characterised as belonging to the

heparin/heparan sulfate family of GAGs and would therefore be expected to bind to similar cell surface molecules as heparin and heparan sulfate.

Immunoprecipitation studies with a limited number of mAbs were used to try and identify the GAG-binding molecules on splenocytes. It was found that with one exception the heparin-binding molecules did not correspond to any of the cell surface antigens tested and, in particular, molecules associated with cell adhesion such as MEL-14, Ly-15 and Pgp-1 (Table 1.1). The exception was a heparin-binding molecule of approx. 40 kDa which was identified as possibly Ly-2. Of particular interest was the finding that the very prominent 90 kDa molecule did not correspond to MEL-14, Ly-15 β-chain or Pgp-1, all of which had been considered as possible candidates due to their molecular masses being approx. 90 kDa.

Interestingly, splenocyte Ly-5 did not bind heparin although as mentioned previously, the thymocyte form binds to a range of SPS including fucoidan, dextran sulfate and heparin (Parish *et al.*, 1988). This is probably due to the presence of different structural types of Ly-5 produced on different cell types, obviously only some of which can bind GAGs.

The Ly-5 family (also called CD45, T200, B220 and leukocyte common antigen family) is a group of glycoproteins expressed on the surface of all mammalian lymphoid and myeloid cells. Members of this family differ in their polypeptide and carbohydrate structures which are expressed differently according to cell type. Variations in N-terminal peptide sequence are coded for by up to three alternately transcribed exons, designated Ex-4, 5, and 6 potentially giving rise to eight distinct forms of mRNA containing 0 to 3 of these exons (Chang *et al.*, 1989), six of which have been isolated so far (Thomas, 1989). For example, thymocytes express the lowest molecular mass form of 180 kDa (do not contain Ex-4, 5 or 6), B cells the highest 220 kDa form (containing Ex-4, 5 and 6) and mature T cells multiple forms (Thomas, 1989; Tung

et al., 1984). Also differences exist in Ly-5 structure due to the state of activation of the T cell. Therefore differences in the heparin-binding capacity of the Ly-5 molecules on splenocytes and thymocytes is not surprising for these lymphocytes carry different forms of Ly-5 on their surface. In fact, it appears that the 180 kDa form on thymocytes which lacks the three N-terminal exons is the heparin-binding species.

It was also demonstrated that the Thy-1 antigen on murine splenocytes did not bind to any of the GAGs tested (Section 4.2.2). However, there is evidence that this molecule does in fact bind to some other SPS such as dextran sulfate and fucoidan (Parish *et al.*, 1988a), which were not tested. Obviously, the endogenous sulfated ligand for this molecule remains to be identified.

Finally, the detection of the very prominent 90 kDa GAG-binding protein on both thymocytes and splenocytes (Figure 4.2) posed an interesting problem, for prior studies performed by Parish *et al.* (1988a), where thymocyte ^{125}I-labelled GAG-binding molecules were examined, did not identify this molecule. This anomaly led to the studies performed in Chapter 5 where attempts were made to characterise further this molecule and thereby resolve the issue.

4.4 SUMMARY

This chapter describes attempts to isolate and characterise GAG-binding molecules on the lymphocyte surface. Initial binding studies with radiolabelled GAGs demonstrated that bovine lung heparin has a binding affinity to splenocytes of approximately 1.1×10^{-6} M. However, due to the large amount of non-specific binding incurred in this assay the binding affinities for other GAG molecules could not be calculated.

Rosetting studies with GAG-coupled red cells demonstrated that between 20% and 90% of lymphocytes express receptors for all the GAGs tested. Analysis of the binding of solubilised radiolabelled cell surface molecules to immobilised GAGs revealed that murine splenocytes express at least 10 distinct cell surface receptors for GAGs (i.e., hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, endogenous splenic-GAGs and heparin) with a wide range of molecular masses (approx. 10-20, 33, 40, 60, 90 and 100 kDa). Thymocytes express similar receptors for GAGs with additional receptor molecules of 190 and 250 kDa. Each GAG bound to a unique profile of cell surface molecules although splenocytes exhibited a much more heterogeneous binding pattern than thymocytes. Immunoprecipitation studies demonstrated that the GAG-binding molecules on splenocytes did not correspond to any of the cell surface antigens tested, namely MEL-14, FcR, CD3, ThB, Ly-5, Ly-15, Pgp-1 and Thy-1, although some data suggested that Ly-2 may bind weakly to heparin.

CHAPTER 5 : CHARACTERISATION OF A 90 KDA GAG-BINDING MOLECULE ON MURINE LYMPHOCYTES : EVIDENCE FOR ASSOCIATION WITH THE LYMPHOCYTE SURFACE VIA AN INOSITOL PHOSPHATE RECEPTOR

5.1 INTRODUCTION

Earlier studies have demonstrated that GAGs can bind to the surface of lymphocytes (Parish *et al.*, 1984, 1988; Parish and Snowden, 1985; Thurn and Underhill, 1986), endothelial cells (Glabe *et al.*, 1983b; Glimelius *et al.*, 1978) and macrophages (Bleiberg *et al.*, 1983; Chong and Parish, 1986) in a specific and saturable manner. However, very few studies have attempted to identify and characterise GAG-binding molecules on the cell surface. An exception was the detection and characterisation of a 56-70 kDa hyaluronic acid-binding protein expressed on the surface of heart fibroblasts (Turley, 1982; Turley *et al.*, 1985). Similar studies by Parish *et al.* (1988) demonstrated that the T200 (Ly-5) antigen displayed on the surface of murine thymocytes is a heparin-binding protein. Additional experiments with a range of SPS revealed that the Thy-1 antigen on murine thymocytes interacts with sulfated carbohydrate structures such as dextran sulfate (Parish *et al.*, 1988), the dextran sulfate possibly mimicking the structure of an endogenous sulfated ligand recognised by Thy-1. Chapter 4 described detailed attempts to detect and identify GAG-binding molecules on the surface of murine lymphocytes. In the course of these studies, a 90 kDa GAG-binding protein was detected on the lymphocyte surface, this molecule being particularly prominent on thymocytes. Interestingly, this 90 kDa GAG-binding species was not detected in membrane preparations of murine thymocytes (Parish *et al.*, 1988), an observation which suggested that the molecule is weakly bound to the lymphocyte membrane and was displaced during the membrane isolation procedure used.

These observations led to the studies performed in this chapter, where attempts were made to further characterise the 90 kDa molecule with particular emphasis being placed on the mode of attachment and release of the molecule from the lymphocyte surface.

5.2 RESULTS

5.2.1 GENERAL PROPERTIES OF THE 90 KDA GAG-BINDING MOLECULE

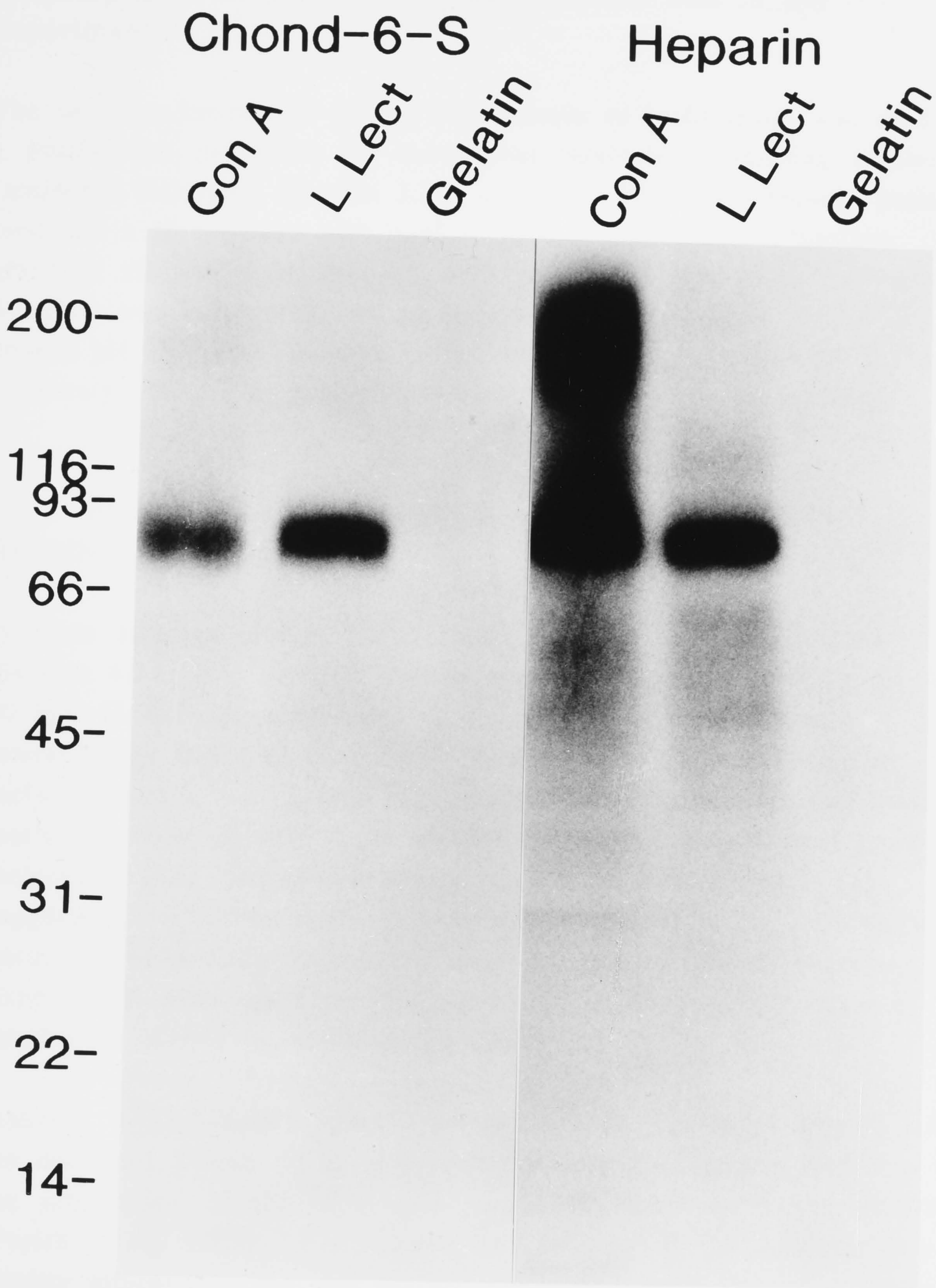
Studies described in Chapter 4 demonstrated that a 90 kDa molecule was one of the prominent cell surface proteins which interacted with GAGs. Before determining the mode of attachment of this molecule to the lymphocyte membrane, some general physicochemical properties of the protein were determined.

Initially, the glycoprotein nature of the 90 kDa molecule was assessed by examining the reactivity of the protein with lectin affinity columns, i.e., Con A and lentil lectin Sepharose. Furthermore, since the 90 kDa molecule was a heparin-binding protein it could be related to FN and thus the ability of the molecule to bind to immobilised gelatin (gelatin-Sepharose) was determined.

In these experiments two ^{125}I-cell surface labelled splenocyte lysates which contained the 90 kDa protein were examined, namely radiolabelled molecules which bound and were eluted from either chondroitin-6-sulfate or heparin-coupled fibres. These molecules were reacted with Con A, lentil lectin or gelatin affinity columns (Section 2.10) and the resultant binding material analysed by SDS-PAGE. The results of such a study (Figure 5.1) showed that the 90 kDa GAG-binding molecule is a glycoprotein containing glucose and/or mannose residues, demonstrated by binding to Con A and lentil lectin, but it was not FN-like as it did not bind to gelatin. Furthermore, other heparin-binding proteins also bound to lentil lectin and especially Con A, although these were not generally seen as discrete bands on the gel. The exception is a heparin-binding molecule of approx. 200 kDa which bound to Con A Sepharose, the identity of which may be the T200 (Ly-5) antigen. Although this molecule was not seen previously in splenocyte lysates, its presence may relate to the activation state of

Figure 5.1 Ability of GAG-Binding Proteins on the Surface of Murine Splenocytes to Bind to Immobilised Lectins and Gelatin

Chondroitin-6-sulfate (Chond6S) or heparin-binding molecules (Heparin) isolated from ^{125}I-labelled murine splenocytes were bound to either Concanavalin A (Con A), lentil lectin (L Lect) or gelatin Sepharose 4B beads and bound material analysed by SDS-PAGE and autoradiography. Exposure time was 5 days. The position of molecular weight markers is indicated in kDa.


Chond-6-S
Heparin
Con A
L Lect
Gelatin
Con A
L Lect
Gelatin
200-
116-
93-
66-
45-
31-
22-
14-

lymphocytes in the spleen of the donor animals used in this experiment.

The selective binding of the 90 kDa protein to lentil lectin was used as a purification procedure for subsequent isoelectric focussing studies. Isoelectric focussing (Section 2.22) of the 90 kDa GAG-binding protein produced a single band with a pI of 6.16 (Figure 5.2). Therefore, although the molecule interacts with polyanions such as chondroitin-6-sulfate and heparin, it has an overall slightly negative charge at neutral pH. Thus, there may be regions of the protein rich in positively charged amino acids which facilitate heparin binding.

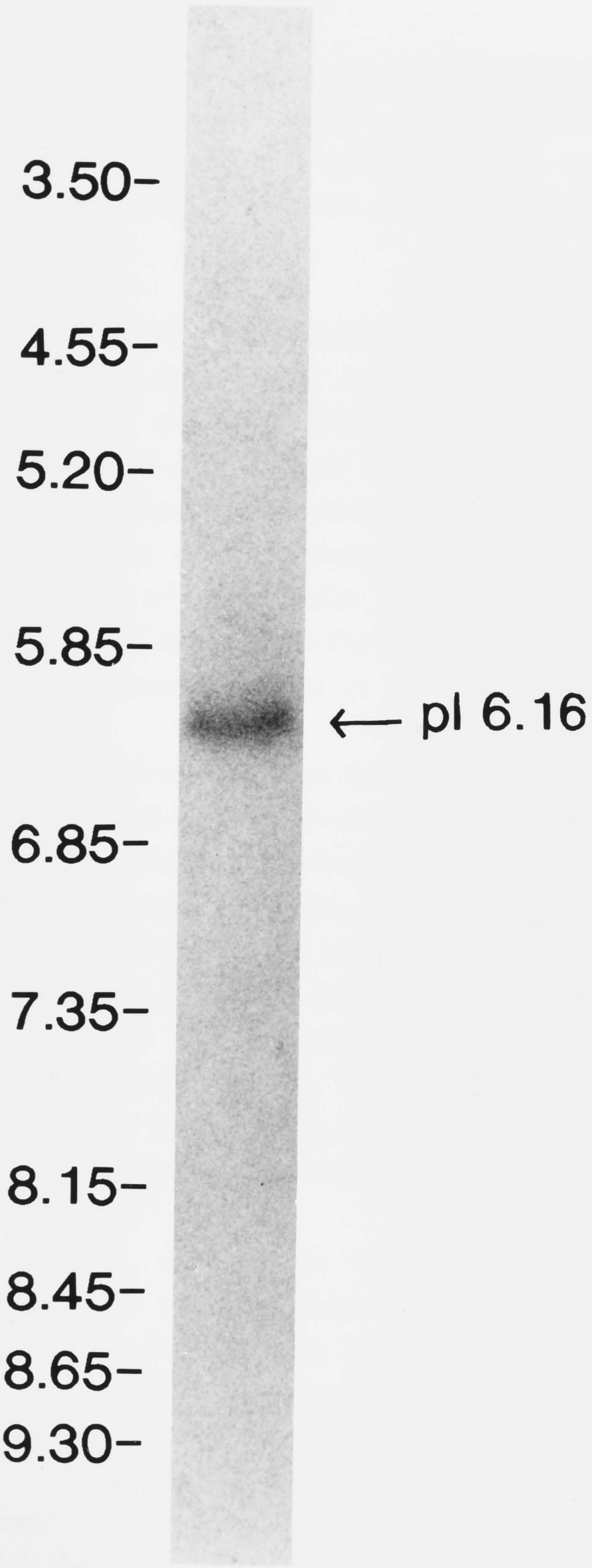
5.2.2 Mode of Association of the 90 kDa GAG-Binding Molecule with the Lymphocyte Surface

Previous sections of this thesis have described the identification (Section 4.2.1) and partial characterisation (Section 4.2.2, 5.2.1) of the 90 kDa GAG-binding molecule on the surface of lymphocytes. Interestingly, this molecule which was present on the thymocyte surface (Section 4.2.1), was not detected in membrane preparations of murine thymocytes (Parish *et al.*, 1988), possibly due to the loss of the molecule during preparation of the thymocyte membranes. This suggested that the molecule may be weakly bound to the cell surface occurring as a peripheral rather than integral membrane protein. Experiments were therefore undertaken to investigate the mode of attachment of this molecule to the cell surface.

Initially, ^{125}I labelled splenocytes and thymocytes were treated with the detergent Tween 40 as this detergent was used as the first step in the preparation of the thymocyte membranes used in earlier studies (Parish *et al.*, 1988). Furthermore, to determine if the molecule was weakly attached to the cell surface via a peripheral linkage system such as an integrin-binding sequence, an ionic interaction and/or a phosphatidylinositol (PI) receptor, ^{125}I labelled lymphocytes were

Figure 5.2 Isoelectric Focussing of the 90 kDa GAG-Binding Protein

The 90kDa protein was purified from an ^{125}I-labelled splenocyte lysate by binding and elution from heparin-coupled fibres followed by lentil lectin affinity chromatography. The isoelectric point was determined as 6.16 by the Phast Gel System when compared with pI standards as indicated.


3.50–
4.55–
5.20–
5.85–
← pI 6.16
6.85–
7.35–
8.15–
8.45–
8.65–
9.30–

treated with RGDS, high salt concentrations or inositol hexaphosphate (phytic acid) respectively (Section 2.11). Previous studies have shown that inositol hexaphosphate releases peripheral cell surface molecules which are probably attached to the cell surface via a PI receptor (Carey and Evans, 1989).

It was found that the 90 kDa GAG-binding molecule was released from the thymocyte (data not shown) and splenocyte surface by treatment of lymphocytes with high salt (2 M KCl) and the detergents Tween 40 and Triton X-100 (Figure 5.3). Release of the molecule from the cell surface by Tween 40 treatment implies that lymphocyte membranes prepared using the Tween 40 procedure (Standring and Williams, 1978) would be depleted of the 90 kDa GAG-binding protein. Furthermore, displacement of the 90 kDa protein by 2 M KCl indicates that the molecule is ionically attached to the cell surface.

Further experiments confirmed the peripheral linkage of the protein. Although the 90 kDa GAG-binding molecule could not be displaced from the cell surface by the integrin-binding sequence RGDS (1 mg/mL), it could be released from the splenocyte (Figure 5.3) and thymocyte (Figure 5.4) surface with inositol hexaphosphate (10 mM). Therefore, the 90 kDa GAG-binding protein appeared to be attached to the lymphocyte surface by a PI receptor. Furthermore, it is evident that the 90 kDa GAG-binding protein is continually being released from the cell surface at a slow rate in PBS alone (control tract; Figure 5.3) as would be expected with a loosely bound molecule.

Following these findings, experiments were performed to further investigate the specificity of interaction between the putative PI receptor and the 90 kDa GAG-binding protein. Initially other possible PI receptor-bound proteins present on the splenocyte surface were identified by examining all proteins displaced from splenocytes by inositol hexaphosphate treatment. Preliminary data depicted in Figure 5.5, showed that splenocytes have a number of proteins which are displaced from the cell surface by inositol hexaphosphate. These

Figure 5.3 Release of 90 kDa GAG-Binding Protein from Splenocytes by Various Treatments

Murine splenocytes were labelled with ^{125}I and cell surface proteins were released by treatment with solutions containing 1 mg/mL RGDS (Arg-Gly-Asp-Ser), 10 mM inositol hexaphosphate (phytic acid; PA), 2.5 M KCl, 2.5% Tween 40, 0.5% Triton X-100 or PBS (Cont). The released proteins were bound to heparin fibres prior to SDS-PAGE analysis. Exposure time of autoradiographs was 4 days. Molecular weight markers are indicated in kDa.

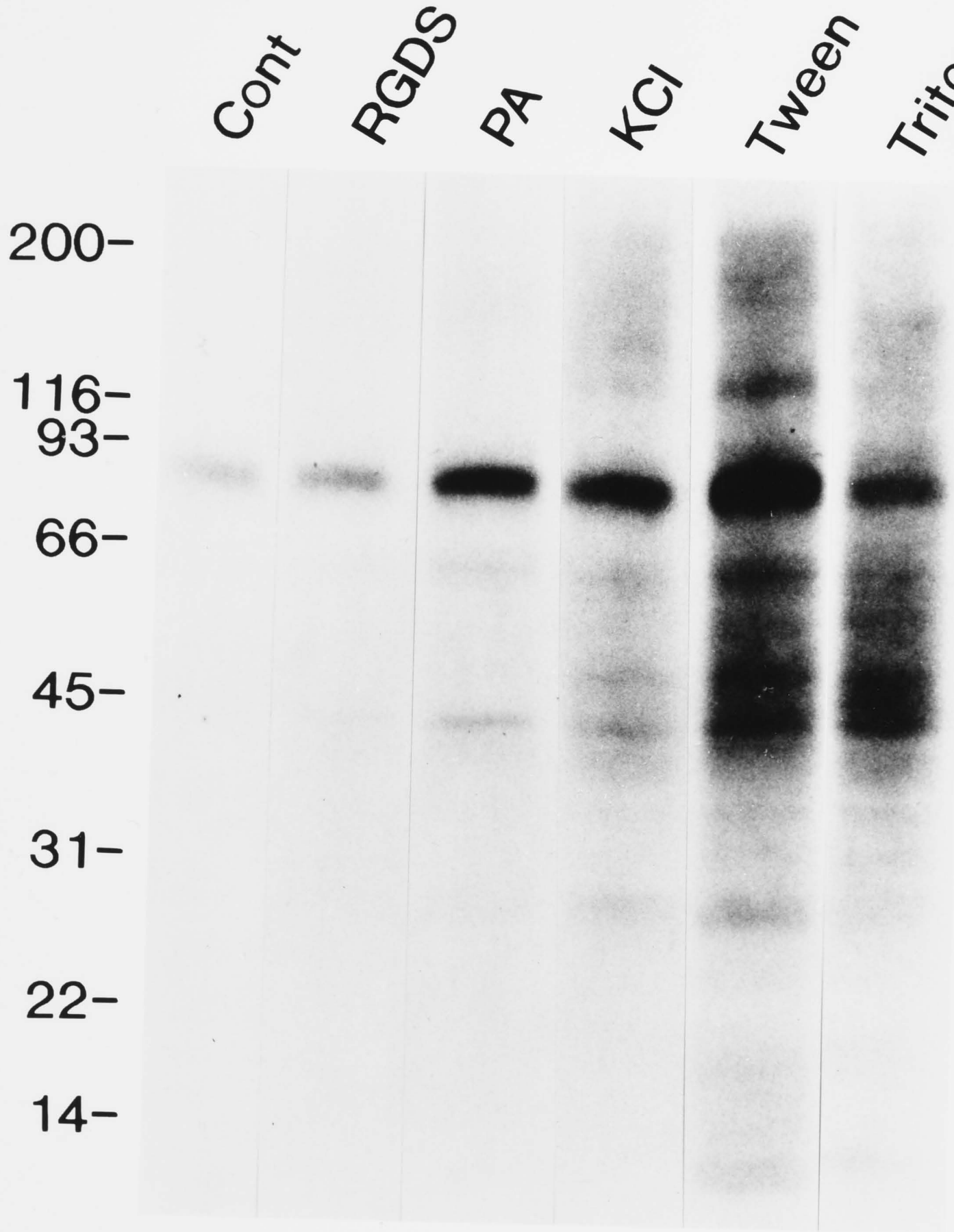

Cont
RGDS
PA
KCl
Tween
Triton
200-
116-
93-
66-
45-
31-
22-
14-

Figure 5.4 Release of 90 kDa GAG-Binding Protein from Murine Thymocytes by Inositol Hexaphosphate Treatment

Murine thymocytes were labelled with ^{125}I and cell surface proteins were released by treatment with either 5 mM inositol hexaphosphate (phytic acid; PA) in PBS or PBS alone (Cont) and then released material bound to heparin fibres prior to analysis by SDS-PAGE. Exposure time of autoradiographs was 7 days. Molecular weight markers are indicated in kDa.

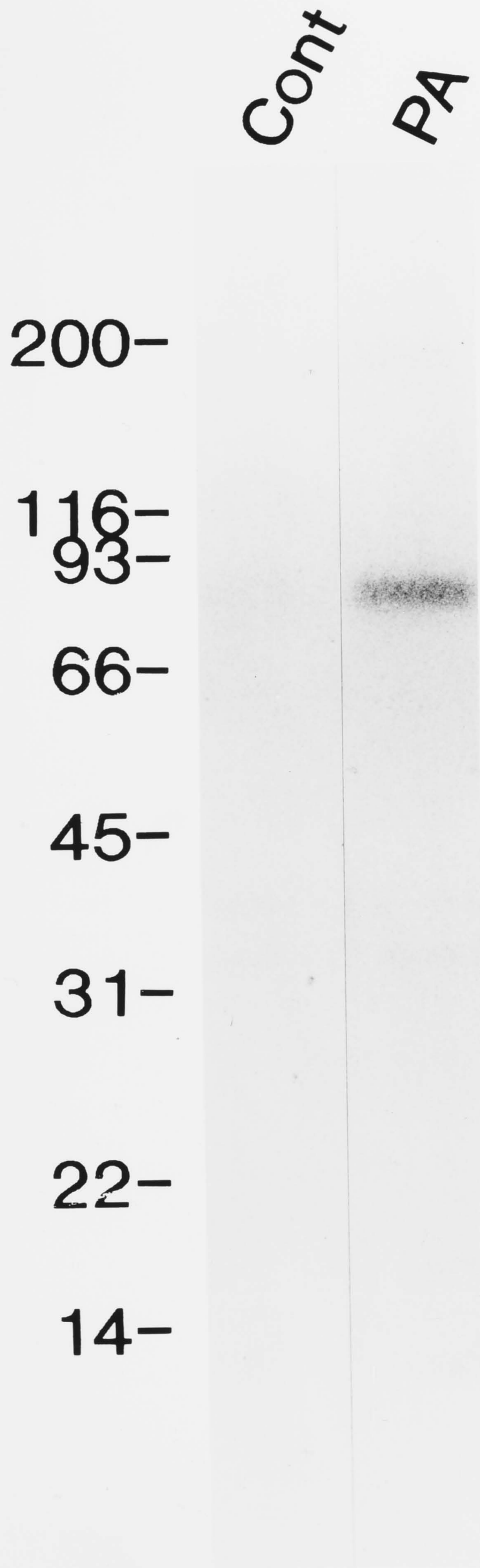
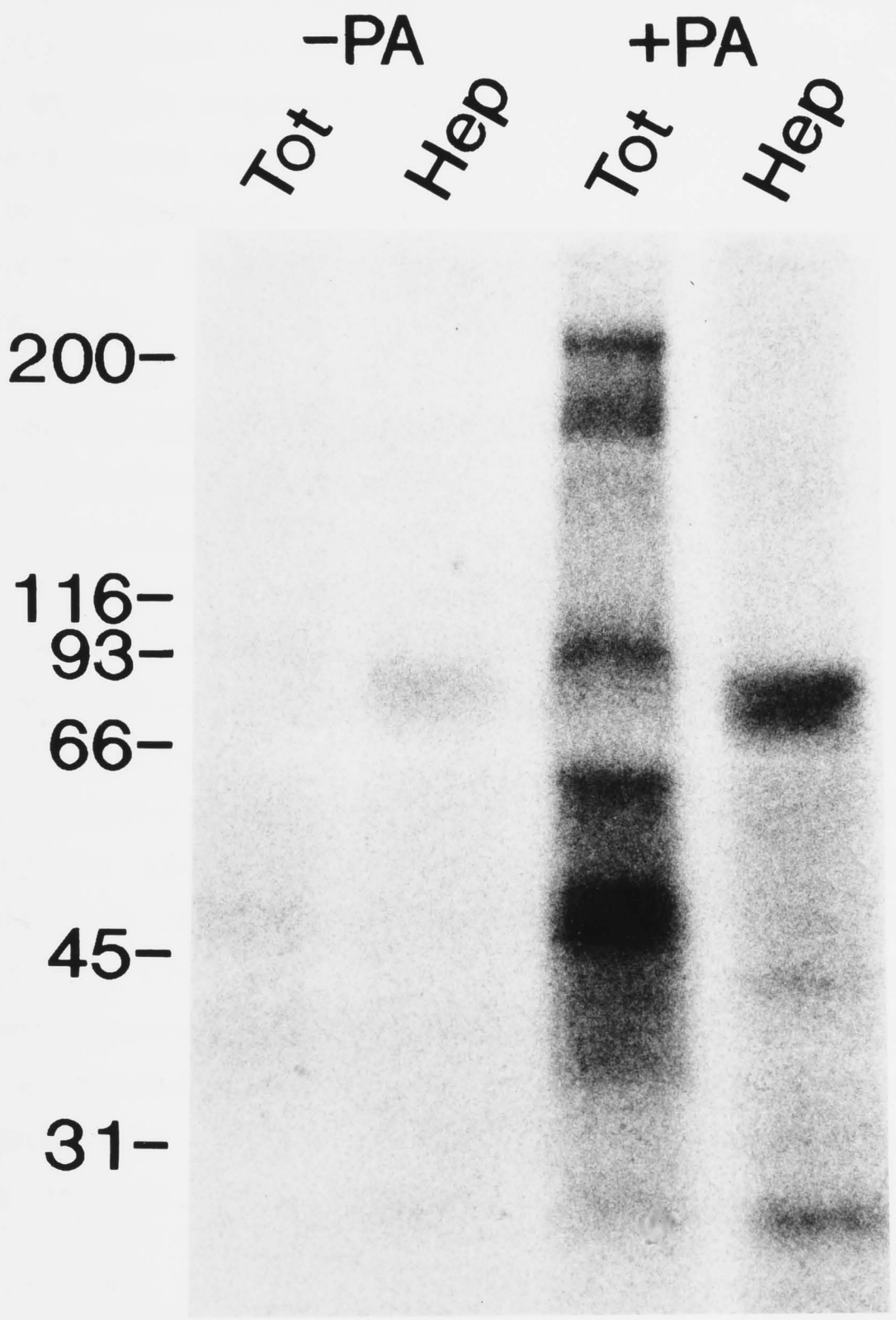

Cont
PA
200-
116-
93-
66-
45-
31-
22-
14-

Figure 5.5 Analysis of Cell Surface Proteins Released from Splenocytes by Inositol Hexaphosphate Treatment

Murine splenocytes were labelled with ^{125}I and cell surface proteins were released by treatment with either 5 mM inositol hexaphosphate (phytic acid; +PA) in PBS or PBS alone (-PA). The released proteins (Tot) and those proteins which bound to heparin fibres (Hep) were then analysed by SDS-PAGE. Exposure time of autoradiographs was 6 days. Molecular weight markers are indicated in kDa.


-PA
+PA
Tot
Hep
Tot
Hep
200-
116-
93-
66-
45-
31-

include an approximately 50 kDa protein and two high MW proteins of around 200 kDa. Two protein bands migrating at around 90 kDa were detected. These bands are probably an artifact as the carrier protein (BSA) used for acetone precipitation of radiolabelled molecules (Section 2.21) migrates in this region of the SDS-PAGE gel and appears to interfere with the migration of the 90 kDa band. This experiment needs to be repeated with other protein carriers in order to resolve this problem. Nevertheless, when inositol hexaphosphate released material was bound to heparin fibres the 90 kDa band was found to bind (Figure 5.5).

Release of the 90 kDa GAG-binding protein from the cell surface by inositol hexaphosphate treatment was found to be very selective. A range of concentrations of inositol hexaphosphate were tested for their ability to release the 90 kDa molecule from the splenocyte surface. It was found that at concentrations as low as 0.31 mM, inositol hexaphosphate released more than twice the amount of 90 kDa protein (222% release of control) than PBS alone (Figure 5.6). Furthermore, inositol derivatives such as inositol hexasulfate, inositol-1-monophosphate and inositol-2-monophosphate at a concentration of 5 mM displaced a much smaller amount of the 90 kDa GAG-binding molecules (187%, 199% and 98%, respectively of PBS control) when compared with inositol hexaphosphate release (724%; Figure 5.7). In fact, when compared with the PBS control, inositol-2-monophosphate did not significantly displace the 90 kDa molecule from the lymphocyte surface.

5.2.3 Reassociation of the 90 kDa GAG-Binding Molecule with Splenocytes

Assuming that the 90 kDa GAG-binding molecule is attached to the lymphocyte surface by a PI receptor (an interaction which can be selectively displaced by inositol hexaphosphate) one would predict that the 90 kDa molecule could reassociate with splenocytes via this

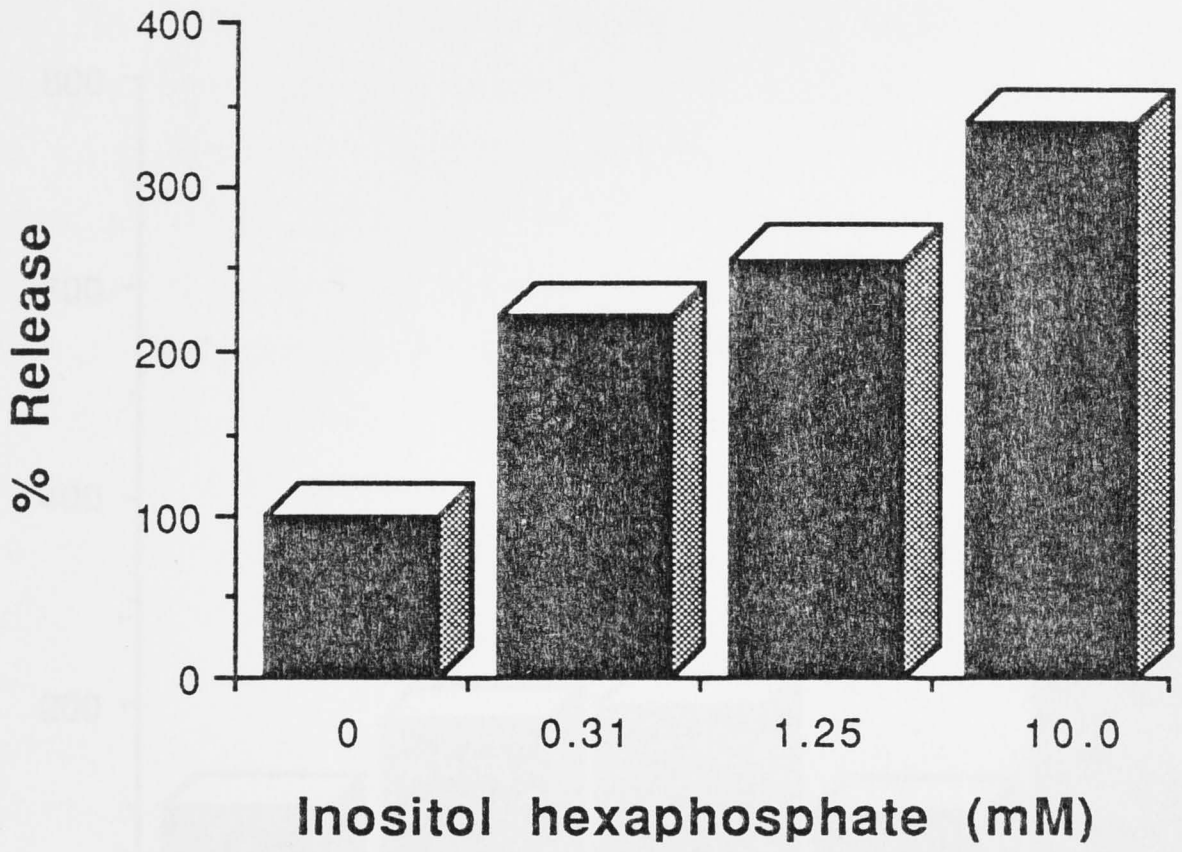


Figure 5.6 Release of 90 kDa GAG-Binding Protein from Splenocytes by Inositol Hexaphosphate

Murine splenocytes were cell surface labelled with ^{125}I and treated with different concentrations of inositol hexaphosphate (0.31, 1.25 and 10.0 mM PA) in PBS or PBS alone. Labelled molecules present in the cell supernatant were then resolved on SDS-PAGE and the amount of 90 kDa protein released, quantified by microdensitometry. The amount of ^{125}I labelled 90 kDa protein released by treatment of the cells with PBS alone was taken as 100%.

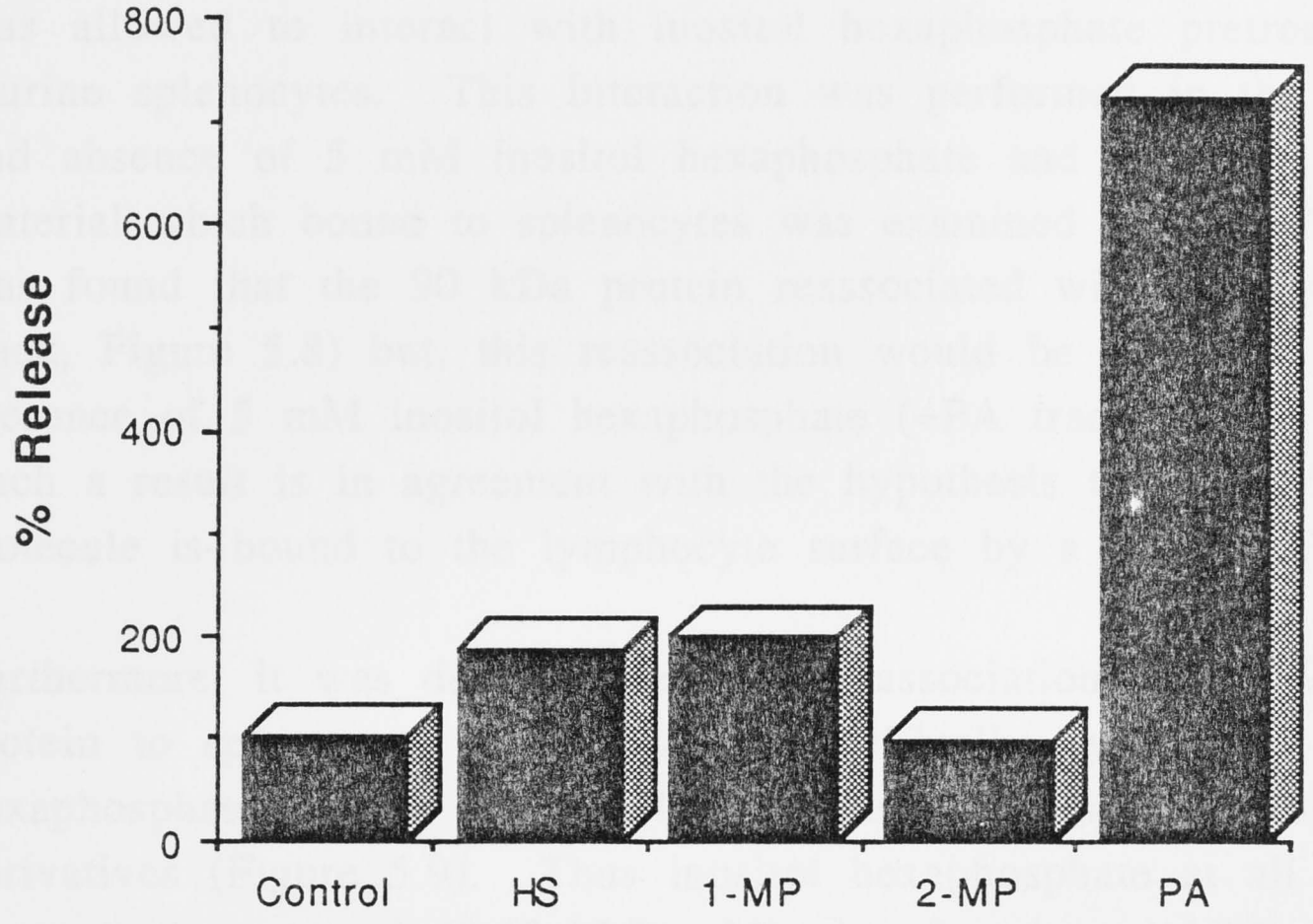


Figure 5.7 Release of 90 kDa GAG-Binding Protein from Splenocytes by Different Inositol Derivatives

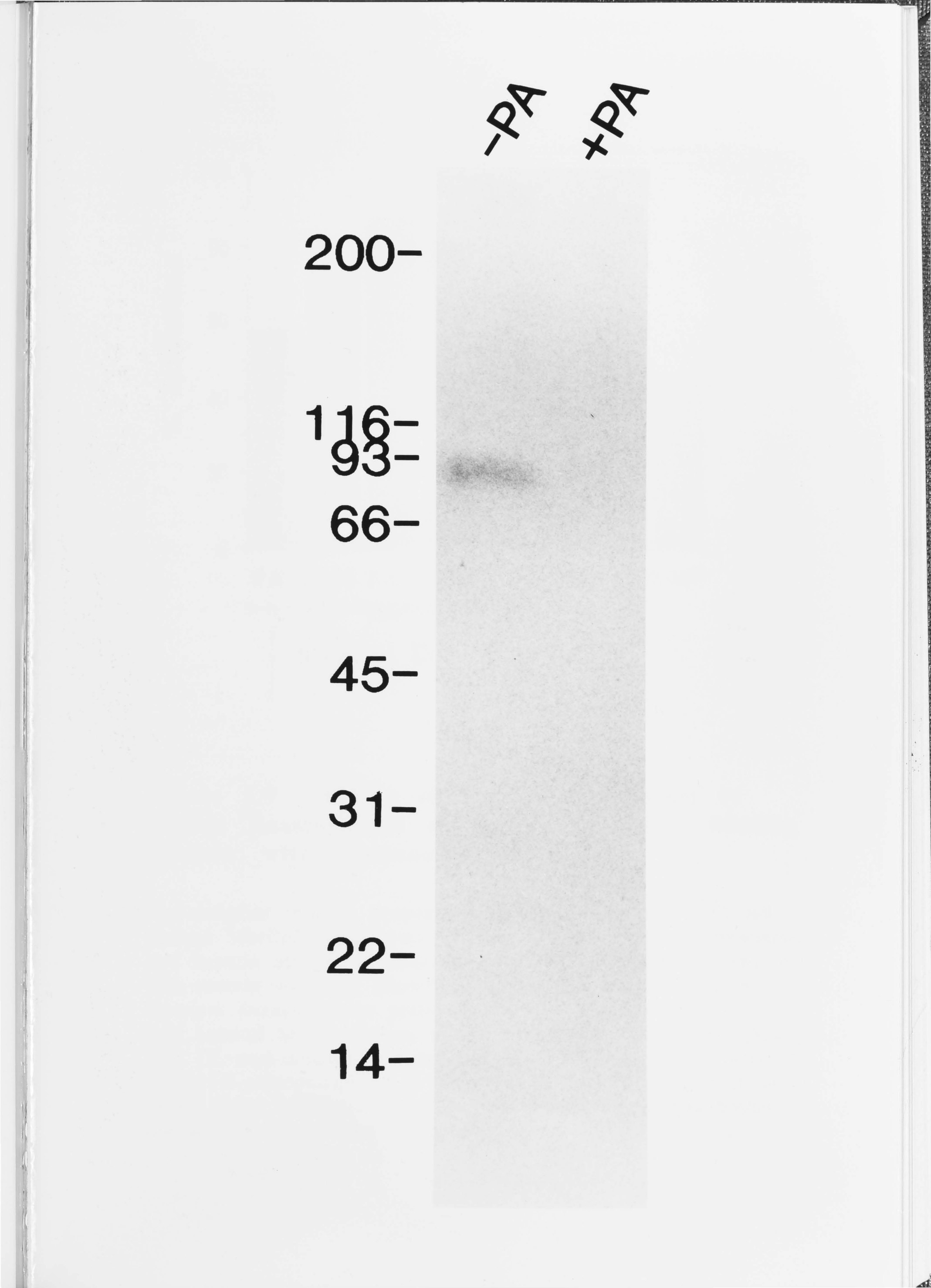
Murine splenocytes were cell surface labelled with ^{125}I and cell surface proteins were released by treatment with solutions of PBS containing 5 mM myo-inositol-hexasulfate (HS), DL-myo-inositol-1-monophosphate (1-MP), myo-inositol-2-monophosphate (2-MP), inositol hexaphosphate (phytic acid; PA) or PBS alone (control). The released proteins were bound to heparin fibres prior to SDS-PAGE and percent release of the 90 kDa protein was calculated by microdensitometry of autoradiographs. The amount of ^{125}I labelled 90 kDa protein released by treatment of the cells with PBS alone was taken as 100%.

receptor. In order to maximise this interaction, spleen cells were pretreated with inositol hexaphosphate to free the PI receptor of endogenous ligands. The ^{125}I-labelled 90 kDa GAG-binding protein released from the splenocyte surface by 5 mM inositol hexaphosphate, was allowed to interact with inositol hexaphosphate pretreated murine splenocytes. This interaction was performed in the presence and absence of 5 mM inositol hexaphosphate and resultant labelled material which bound to splenocytes was examined by SDS-PAGE. It was found that the 90 kDa protein reassociated with splenocytes (-PA track, Figure 5.8) but, this reassociation would be inhibited by the presence of 5 mM inositol hexaphosphate (+PA track, Figure 5.8). Such a result is in agreement with the hypothesis that the 90 kDa molecule is bound to the lymphocyte surface by a specific PI receptor.

Furthermore, it was demonstrated that reassociation of the 90 kDa protein to splenocytes was inhibited specifically by inositol hexaphosphate, but to a much lesser extent by other inositol derivatives (Figure 5.9). Thus inositol hexaphosphate at all concentrations tested (0.63-10.0 mM) was found to inhibit reassociation by greater than 50%, with 10 mM inositol hexaphosphate inhibiting reassociation by 96%. In contrast, 5 mM inositol hexasulfate, inositol-1-monophosphate and inositol-2-monophosphate inhibited reassociation by only 42, 12 and 45% respectively, further demonstrating the specificity of interaction between the 90 kDa molecule and its receptor. The reason for the reversal of the effects seen with inositol-1-monophosphate and inositol-2-monophosphate between this experiment and earlier displacement experiments (Figure 5.7 and 5.9) is unknown.

Figure 5.8 Reassociation of the 90 kDa GAG-Binding Protein with Splenocytes

Murine splenocytes were labelled with ^{125}I and the 90 kDa protein was released by treatment with 5 mM myo-inositol hexaphosphate (phytic acid) in PBS. The released proteins were then allowed to reassociate with unlabelled, phytic acid-depleted splenocytes in the absence (-PA) or presence (+PA), of 5 mM phytic acid and the resultant splenocyte lysates analysed by SDS-PAGE. Exposure time was 16 days. Molecular weight markers are indicated in kDa.


-PA
+PA
200-
116-
93-
66-
45-
31-
22-
14-

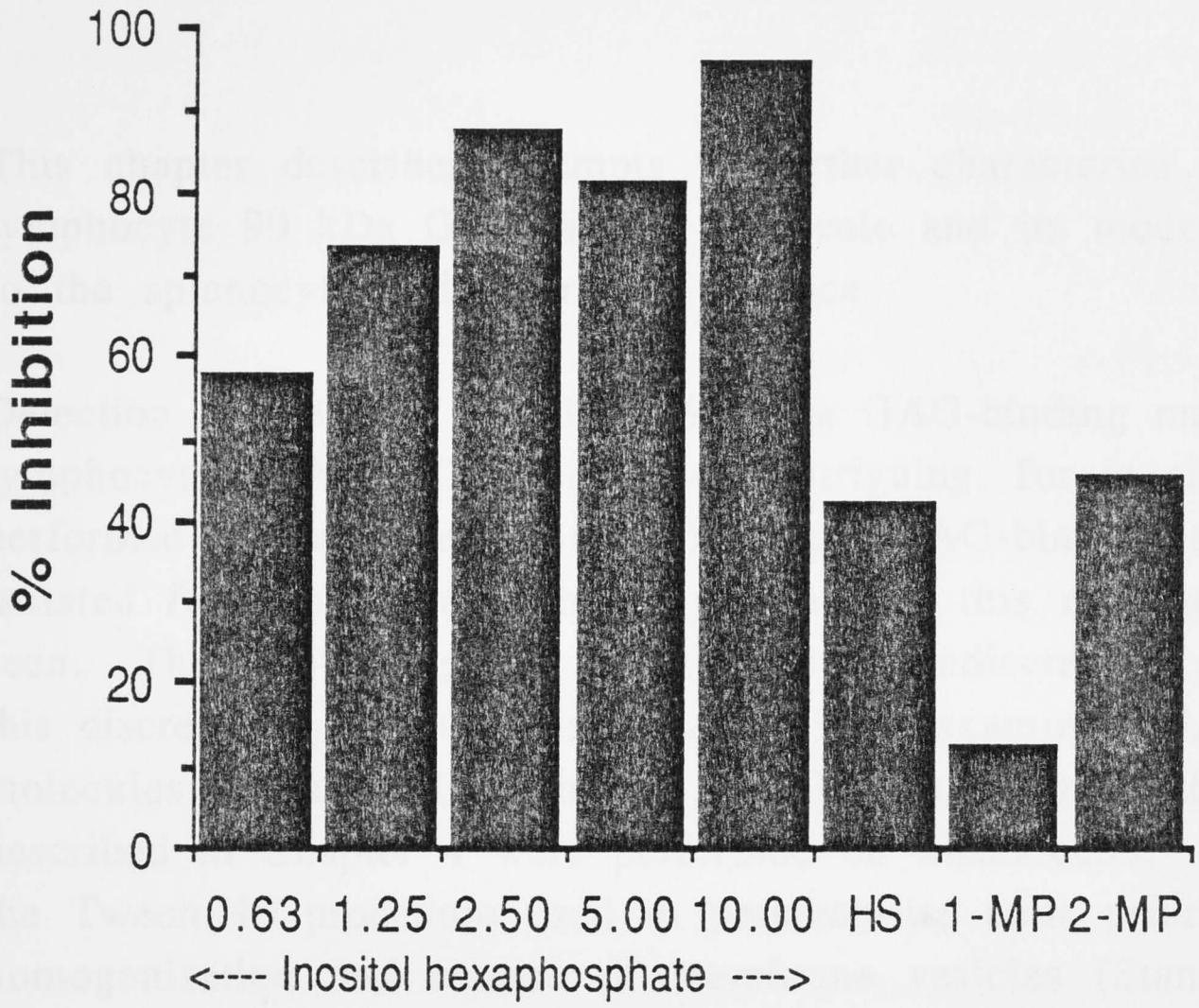


Figure 5.9 Ability of Inositol Derivatives to Inhibit Reassociation of the 90 kDa GAG-Binding Protein with Splenocytes

Radiolabelled 90 kDa protein was semi-purified from ^{125}I cell surface labelled splenocytes by inositol hexaphosphate treatment and heparin affinity chromatography of the supernatant. The 90 kDa protein was then allowed to reassociate with splenocytes (inositol hexaphosphate pretreated) in the presence of 0.63-10.0 mM inositol hexaphosphate, 5 mM myo-inositol-hexasulfate (HS), 5 mM DL-myo-inositol-1-monophosphate (1-MP) or 5 mM myo-inositol-2-monophosphate (2-MP) and the percent inhibition of reassociation calculated based on cpm bound, using cpm bound in the absence of inhibitor as the control.

5.3 DISCUSSION

This chapter describes attempts to further characterise the murine lymphocyte 90 kDa GAG-binding molecule and its mode of attachment to the splenocyte and thymocyte surface.

Detection of the very prominent 90 kDa GAG-binding molecule on the lymphocyte surface (Chapter 4) was intriguing, for in similar studies performed by Parish *et al.* (1988), where GAG-binding molecules were isolated from the murine thymocyte surface, this molecule was not seen. The studies reported in this chapter indicate that the reason for this discrepancy is that Parish *et al.* (1988) examined GAG-binding molecules in isolated thymocyte membranes whereas the experiments described in Chapter 4 were performed on intact cells. It appears that the Tween 40 procedure used to permeabilise cells prior to homogenisation and release of membrane vesicles (Standring and Williams, 1978) solubilises many membrane molecules, particularly peripheral membrane proteins such as the 90 kDa GAG-binding molecule. This point is clearly evident in Figure 5.3 where Tween 40 treatment of splenocytes was as effective as Triton X-100 at solubilising membrane proteins. The rationale behind the Tween 40 membrane isolation procedure is that Tween 40 is a "weak" detergent which merely permeabilises plasma membranes so that they are more readily released from the cell by homogenisation. However, the experiments described in this chapter indicate that this assumption cannot be made, particularly when isolation and characterisation of peripheral membrane proteins is being considered.

Since the 90 kDa GAG-binding protein appeared to be a peripheral membrane protein the mode of attachment of the molecule to the lymphocyte surface was investigated. It was found that the molecule was not FN-like (did not bind gelatin). Furthermore the protein was probably not related to any cell surface receptors of the integrin family, which interact with RGD sequences on cell adhesive proteins

(i.e., FB, VWF, VN, osteopontin, collagens and thrombospondin), as the peptide RGDS failed to release the 90 kDa molecule from the lymphocyte surface (Figure 5.3). However, release of the molecule by 2 M KCl suggested some form of ionic interaction between the 90 kDa molecule and the cell surface.

Several lines of evidence suggest that the 90 kDa molecule is bound to the lymphocyte surface by a PI receptor. Firstly, the molecule is the predominant species displaced from the lymphocyte surface by inositol hexaphosphate treatment at concentrations as low as 0.31 mM (Figure 5.6) a treatment used by others to displace molecules from cell surface PI receptors (Ishihara *et al.*, 1987). Secondly, displacement of the 90 kDa molecule from the lymphocyte surface by inositol hexaphosphate was highly specific as similar concentrations of other inositol derivatives such as inositol hexasulfate, inositol-1-monophosphate and inositol-2-monophosphate were either much less effective or ineffective at displacing the molecule from the cell surface (Figure 5.7). Furthermore, this data suggests that the molecule was not displaced from the cell surface by ionic interactions alone, for inositol hexasulfate has a similar charge density to inositol hexaphosphate at pH 7. In fact, inositol hexaphosphate, which has a range of pK_a values from 1.9 to 9.5 has at pH 7 and at a similar ionic strength to our experiments (0.2 M KCl) a net negative charge of approx. 7.8 (Evans *et al.*, 1982). Similarly, inositol hexasulfate, a derivative of the strong acid H_2SO_4, would be totally ionised at pH 7 with a net negative charge of six. Therefore although both molecules have similar charge densities, their propensity to release the 90 kDa molecule is very different, a further indication that displacement is specific. It is therefore likely that steric factors may play an important role in this process.

Other evidence also supports the view of a PI receptor for the 90 kDa GAG-binding protein. The 90 kDa GAG-binding protein released from lymphocytes by inositol hexaphosphate treatment could reassociate with splenocytes *in vitro*. This interaction could be virtually

completely inhibited by inositol hexaphosphate whereas, inositol hexasulfate, inositol-1-monophosphate and inositol-2-monophosphate were much less effective at inhibiting reassociation (Figure 5.9), a finding which again indicates the specific nature of the association of the 90 kDa protein with the lymphocyte surface.

Another interesting feature of anchorage of the 90 kDa protein to the lymphocyte surface is the observed "background" release of molecules into the cell supernatant under physiological conditions (Figure 5.3, control tract), reinforcing the idea of a reversible interaction between the PI receptor and the 90 kDa molecule.

To date only one other study has reported the association of molecules with the cell surface via a PI receptor (Ishihara *et al.*, 1987). This study found that rat hepatocytes synthesise a heparan sulfate proteoglycan which appears to be bound to the plasma membrane via a PI receptor, an interaction which can be disrupted specifically by inositol hexaphosphate. There are however, many studies demonstrating that a wide range of cell surface molecules are directly anchored to the cell membrane via glycosyl-phosphatidylinositol (GPI) structures. Examples of lymphocyte cell surface antigens in this category are Thy-1, Qa, ThB, RT-6, Ly-6, T-cell activating protein and LFA-3 (reviewed by Ferguson and Williams, 1988; Low, 1989). GPI anchors on different proteins share a number of major structural features namely, the protein is linked to the GPI anchor through the C-terminal amino acid via a phosphoethanolamine moiety and a glycan (containing mannose and glucosamine). Variations in this general structure include additional sugar residues or phosphoethanolamines and changes in the linkage structure. Obviously the presence of phosphoethanolamine, ubiquitous to all GPI anchors, would provide an ideal method for further confirming the anchorage form of the 90 kDa protein to the lymphocyte surface. Experiments are planned where the incorporation of ^{3}H-ethanolamine into the 90 kDa protein will be assessed and the ability of this radiolabelled molecule to reassociate with splenocytes tested.

Additional evidence for the PI receptor could be obtained by attempting to displace and/or inhibit reassociation of the 90 kDa protein with PI-PLC (phosphatidylinositol-specific phospholipase C) released proteins from murine thymocytes. Such an experimental approach would also provide possibilities for characterising the endogenous GPI structure recognised by the PI receptor.

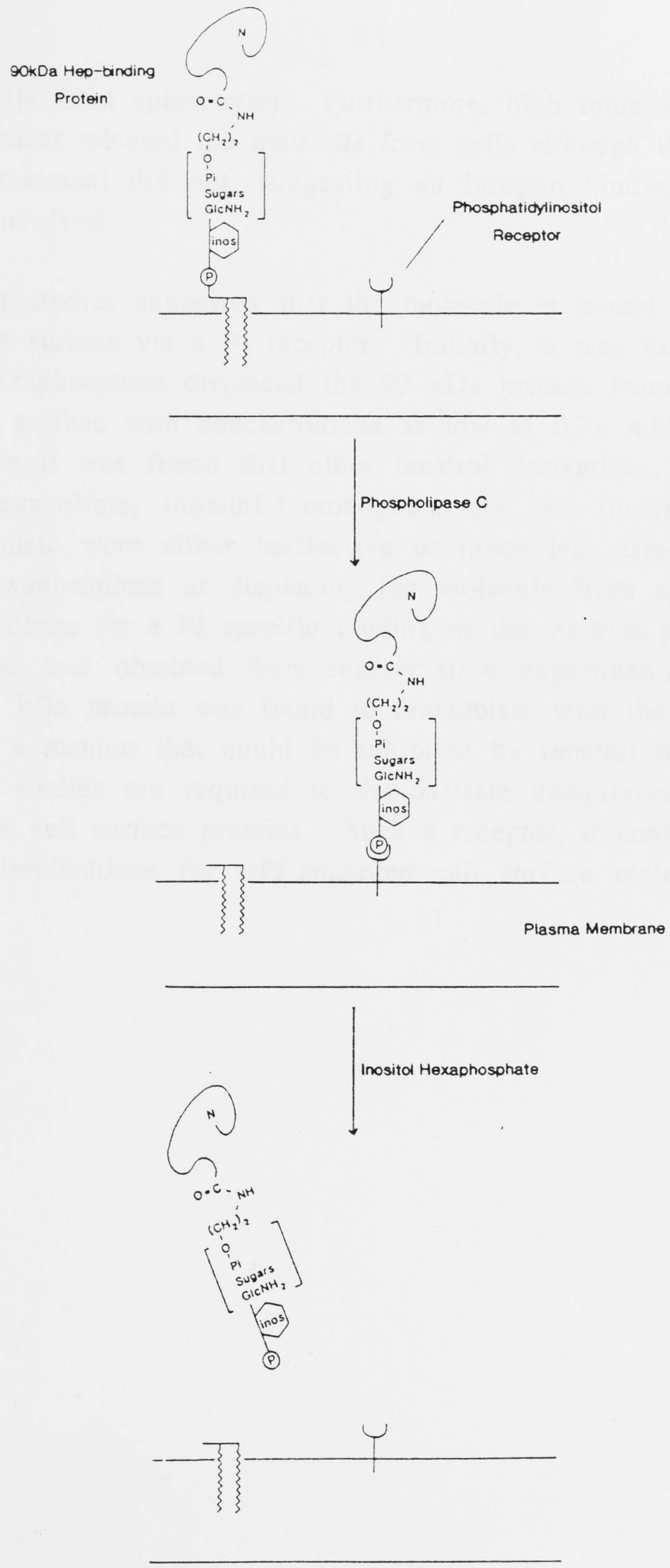
The possible presence of a PI receptor on lymphocytes led to the development of a model for the attachment of the 90 kDa GAG-binding molecule to the cell surface (Figure 5.10), similar to that reported for the membrane anchoring of heparan sulfate proteoglycans (Ishihara *et al.*, 1987). The 90 kDa GAG-binding protein is possibly linked to the cell surface initially via a GPI anchor, which is cleaved by endogenous phospholipase C resulting in release of the 90 kDa protein and its subsequent reattachment to the cell surface via the PI receptor. The functional feasibility and significance of this process would need to be investigated further, although one possible explanation for this process is that it may function to internalise GPI linked cell surface molecules (Ishihara *et al.*, 1987).

5.4 SUMMARY

Earlier studies (Chapter 4) demonstrated the presence of a prominent 90 kDa GAG-binding protein on the surface of murine lymphocytes. Further characterisation of the 90 kDa GAG-binding molecule revealed that it is a glycoprotein with a slightly acidic pI (pI 6.16). The failure to detect this molecule in Tween 40 isolated membranes of murine thymocytes suggested that the 90 kDa molecule may be a peripheral rather than an integral membrane protein. Studies described in this chapter confirmed such a hypothesis as the 90 kDa protein was found to be spontaneously released from the lymphocyte surface at a significant rate and Tween 40 treatment released large quantities of

Figure 5.10 Hypothetical Model for the Attachment of the 90 kDa GAG-Binding Protein to the Lymphocyte Surface

The 90 kDa GAG-binding protein is anchored to the lymphocyte surface by means of a phosphatidylinositol anchor fatty acid chain which is embedded in the plasma membrane. This anchor when cleaved by endogenous phospholipase C releases the 90 kDa molecule which can then bind to the cell surface via a phosphatidylinositol receptor. Treatment of the cells with inositol hexaphosphate (phytic acid) selectively displaces this molecule from the cell surface.


90kDa Hep-binding
Protein
N
O=C
NH
(CH2)2
O
Pi
Sugars
GlcNH2
inos
P
Phosphatidylinositol
Receptor
Phospholipase C
Plasma Membrane
Inositol Hexaphosphate

the molecule from splenocytes. Furthermore, high ionic strength (2 M KCl) treatment released the molecule from cells although RGDS (1 mg/mL) treatment did not, suggesting an integrin binding sequence was not involved.

Subsequent studies suggested that the molecule is bound to the lymphocyte surface via a PI receptor. Initially, it was found that inositol hexaphosphate displaced the 90 kDa protein from the splenocyte surface with concentrations as low as 0.31 mM. Furthermore it was found that other inositol derivatives, namely inositol hexasulfate, inositol-1-monophosphate and inositol-2-monophosphate were either ineffective or much less effective than inositol hexaphosphate at displacing the molecule from splenocytes. Further evidence for a PI specific binding of the 90 kDa protein to lymphocytes was obtained from reassociation experiments where the soluble 90 kDa protein was found to reassociate with the lymphocyte surface in a manner that could be inhibited by inositol hexaphosphate. Additional studies are required to demonstrate unequivocally a PI receptor for cell surface proteins. Such a receptor, if confirmed, has important implications for GPI-anchored cell surface molecules.

CHAPTER 6 : ANALYSIS OF GAG RECEPTORS ON LYMPHOMA CELL LINES WITH DIFFERENT HOMING PROPERTIES

6.1 INTRODUCTION

Previous studies have indicated that SPS recognition probably plays an important role in lymphocyte migration (Section 1.7). Direct evidence for *in vivo* involvement has included the finding that certain SPS such as dextran sulfate and heparin when injected into animals cause leucocytosis and inhibit lymphocyte recirculation (Bradfield and Born, 1974; Jansen *et al.*, 1962; Sasaki and Suchi, 1967). Also HEV can rapidly incorporate radiolabelled sulfate into a glycolipid which is secreted and causes lymphocytes to localise at intradermal sites of injection (Andrews *et al.*, 1982, 1983). Furthermore, fucoidan, a sulfated polymer of L-fucose, was found to be a potent inhibitor of lymphocyte adhesion to HEV *in vitro* (Stoolman *et al.*, 1987; Stoolman and Rosen, 1983), being as effective as the phosphomannan polymer PPME. However, the most conclusive *in vivo* evidence for SPS involvement in lymphocyte recirculation comes from the work of Brenan and Parish (1986) which demonstrated the selective effects of SPS on entry, displacement and furthermore positioning of lymphocytes within lymphoid organs (Section 1.7.3).

Chapters 4 and 5 described detailed attempts to identify and characterise GAG-binding molecules on the surface of murine lymphocytes and in particular, describe some interesting molecular features of a prominent 90 kDa GAG-binding protein. In this final series of experiments attempts were made to determine the functional significance of GAG-binding molecules on lymphocytes in terms of lymphocyte splenic-homing *in vivo*. Lymphoma cell lines were chosen with specific migratory preference for the spleen in the hope of identifying ubiquitous GAG-binding molecules which may be involved in splenic entry and positioning. Of particular interest was the involvement of the 90 kDa GAG-binding protein, identified and partially characterised in Chapters 4 and 5, in lymphocyte migration to the spleen.

6.2 RESULTS

6.2.1 MIGRATION BEHAVIOUR OF LYMPHOMA CELL LINES

Initially, in order to determine possible candidate molecules which may be involved in splenic-homing a number of lymphomyeloid cell lines (Table 2.2; Section 2.4) were examined for their ability to enter murine spleen (Section 2.23). Eleven lymphoma cell lines and one mastocytoma line were labelled with the intracellular fluorescent dye H33342 (Section 2.23) and injected intravenously into recipient mice (Table 2.2). Spleens were removed 2 h post injection and cell line splenic entry determined by examining spleen cell suspensions and splenic sections for their content of fluorescent cells (Table 6.1). It was found that seven cell lines failed to enter the spleen (i.e., EL-4, BL/VL3, MBL-2, RK4.7, C6VL/1, P815 and LSTRA) whereas five lines gained access (i.e., BCL.1, $RD10_S$, $R1^+$, $R1^-$ and CL2-FT2). Interestingly, the ability of the lines to enter the spleen was not related to either their cellular origin, as both T cell and B cell lines entered, or their mode of induction and propagation (i.e., *in vivo* or *in vitro*)(Table 2.2; Table 6.1). Furthermore, both $R1^+$ and $R1^-$ cell lines entered the spleen, implying that class I MHC antigens are not involved in splenic entry as these two cell lines only differ in H-2 antigen expression, i.e., $R1^-$ is a variant of $R1^+$ which has been selected for lack of cell surface H-2 antigens (Hyman and Stallings, 1976).

Of the five cell lines which exhibited splenic entry, three were chosen for further study. These were BCL.1 and $RD10_S$, both B cell lymphomas maintained *in vivo* and $R1^+$ a thymoma propagated *in vitro*. Both BCL.1 and $RD10_S$ grow as splenic tumours in recipient mice, large tumours appearing in the spleen 14-16 days after the injection of 2×10^6 and 10^6 cells respectively (Figure 6.1). EL-4, a thymoma maintained by *in vitro* culture, was used as a control cell line which failed to enter the spleen.

Figure 6.1 Splenic Tumours

Splenic tumours resulting from the injection of either (a) 2 x 10^6 BCL.1 cells intraperitoneally or (b) 10^6 $RD10_S$ cells intravenously 14 and 16 days earlier, respectively. A normal murine spleen is included in each figure for comparison.

(a)

(b)

Table 6.1 Ability of Different Cell Lines to Enter Spleen

Cell lines failing to enter spleen	Cell lines entering spleen
EL-4 (thymoma)	BCL.1 (B cell lymphoma)
BL/VL3 (thymoma)	$RD10_S$ (B cell lymphoma/hybridoma)
MBL-2 (thymoma)	RI^+ (thymoma)
RK4.7 (pre T cell)	RI^- (thymoma)
C6VL/1 (thymoma)	CL2-FT2 (thymoma)
P815 (mastocytoma)	
LSTRA (thymoma)	

H33342-labelled cells (2×10^{7}) were injected intravenously into recipient mice and spleens removed 2 h post injection. Splenic entry of fluorescent cells was determined by fluorescence microscopy.

As well as quantifying the entry of the four cell lines into the spleen, the migration of these cell lines to other organs, particularly lymphoid organs such as PLN, PP and MLN, was examined. Table 6.2 presents the results of such an analysis, each cell line being labelled with the fluorescent dye H33342 and 2 h following intravenous injection, the entry of fluorescent cells into each organ determined. In order to gain some measure of the efficiency of organ entry the data is presented as percentage of entry relative to H33342 labelled splenocytes. Results depicted in Table 6.2 show that the entry of $R1^+$, BCL.1 and $RD10_S$ into spleen relative to splenocytes was 9.3, 33.6 and 62.8% respectively, compared to negligible entry by EL-4. Entry of the cell lines into other lymphoid organs, namely PLN, PP and MLN, was found to be unrelated to splenic entry. For example, $R1^+$ and $RD10_S$ did not appreciably enter PLN but both entered spleen. Furthermore, PLN and PP entry did not appear to be related, as shown by the migration of $RD10_S$ to PP but not to PLN. In contrast, MLN entry appeared to occur as a combination of both PLN and PP entry as would be expected from earlier specificity of migration studies (Section 1.5).

Table 6.2 Entry of H33342 Labelled Lymphoma Cell Lines into Different Organs

Cell line	% Entry relative to splenocytes					
	Spleen	Peripheral lymph node	Peyers patch	Mesenteric lymph node	Liver	Lungs
EL-4	0.5 ± 0.3	0	0	0	157.9 ± 22.1	66.7 ± 20.9
$R1^+$	9.3 ± 2.0	0	7.9 ± 3.5	1.2 ± 1.2	321.1 ± 34.7	266.7 ± 53.3
BCL.1	33.6 ± 5.8	6.9 ± 0.5	25.6 ± 6.7	54.2 ± 6.2	215.8 ± 30.5	377.8 ± 33.3
$RD10_S$	62.8 ±10.9	1.2 ± 0.5	174.4 ± 11.8	45.3 ± 3.6	73.7 ± 23.2	55.6 ± 17.6

H33342-labelled cells (2×10^7) were injected into recipient mice and organs removed 2 h post injection.
Mean and standard errors were calculated from five counts taken from each of three mice (spleen) or one mouse (all other organs).

Entry of the lymphoma cell lines into the liver and lungs was also quantified to determine if trapping of cells in these organs indirectly affected splenic entry. Although, some lines were trapped in the liver and lungs more effectively than normal splenocytes this did not correlate with lack of splenic entry. For example, there was less trapping of the non-splenic homing cell line EL-4 in the liver and lungs than the lines $R1^+$ and BCL.1 which entered the spleen.

To analyse further splenic migration *in vivo*, the positioning of fluorescently labelled cells was visualised by fluorescence microscopy of whole spleen sections. Figure 6.2 depicts a typical example of this where localisation of H33342-labelled cells in splenic sections was examined. It can be seen that the lymphoma cell lines $R1^+$, BCL.1 and $RD10_S$ and normal splenocytes entered the spleen. Also it appears from these photos and many others not shown, that each of these cell lines ($R1^+$, BCL.1 and $RD10_S$) migrates into the red pulp and marginal zones of the spleen and probably few cells enter the white pulp. This localisation pattern contrasts with that of normal splenocytes where migration of fluorescent cells into the white pulp is clearly evident 2 h post injection (Figure 6.2). The white pulp is easily identifiable in splenic sections as it autofluoresces to a greater extent than other splenic tissue (Figure 6.2, control). Other studies where sections were taken at 8 and 24 h post injection (data not shown) indicate similar patterns of splenic localisation of the lymphoma cell lines, although the numbers of cells had decreased 24 h post injection. Finally, EL-4 acted as a control in these experiments, this cell line failing to enter the spleen (Figure 6.2) as expected from earlier studies (Table 6.2).

6.2.2 Identification of GAG-Binding Molecules on Lymphoma Cell Lines

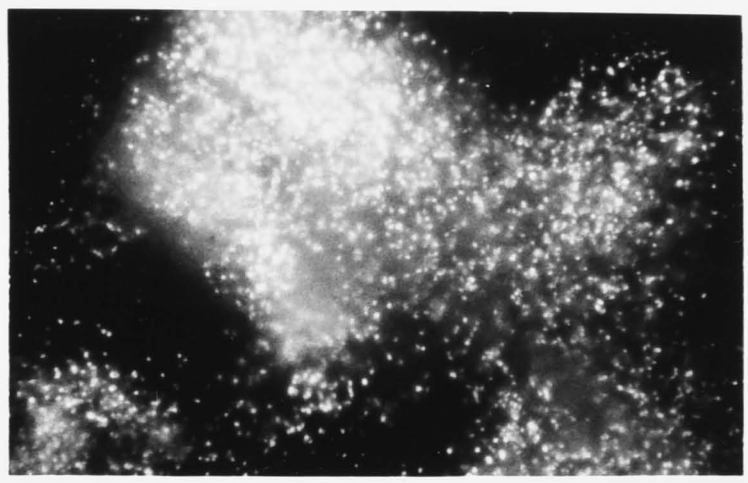
Following the identification of lymphoma cell lines that migrated to the spleen, attempts were made to identify GAG-binding molecules on these cells which may be involved in splenic entry. ^{125}I-labelled cell

Figure 6.2 Positioning of Lymphoma Cell Lines in Spleen

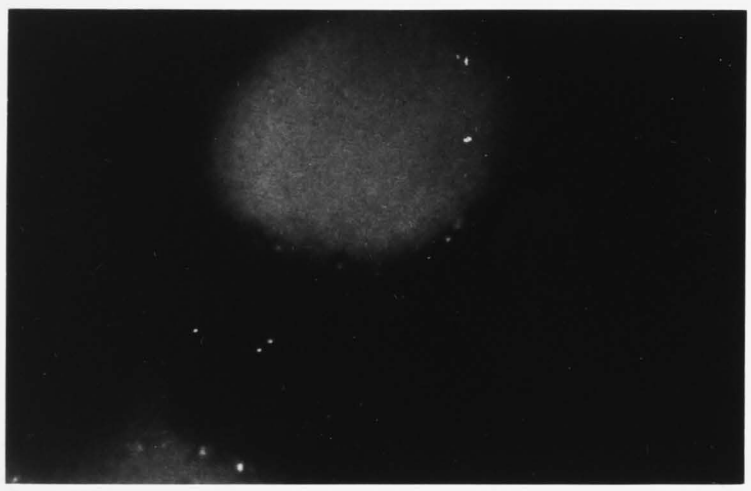
Positioning of H33342-labelled lymphoma cell lines EL-4, $R1^+$, BCL.1 and $RD10_S$ compared with H33342-labelled splenocytes in spleen at 2h after intravenous injection of 2×10^7 fluorescently labelled cells into syngeneic recipient mice. Control represents splenic section from an uninfected animal and depicts the autofluorescence of the white pulp of the spleen.

Control

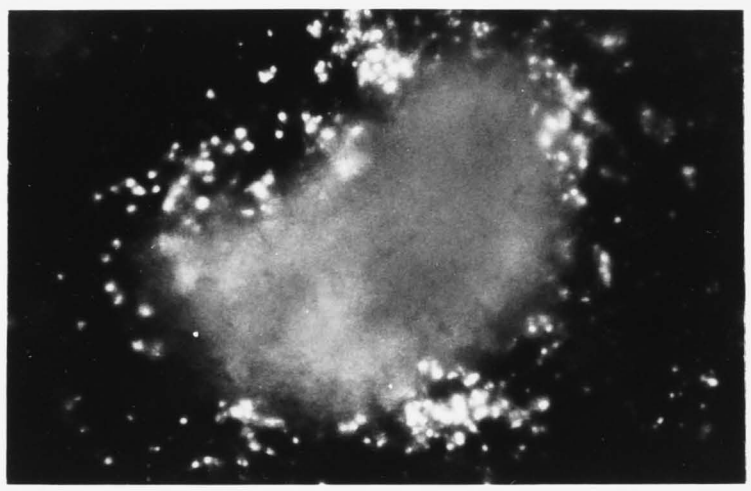
Splenocyte



EL-4

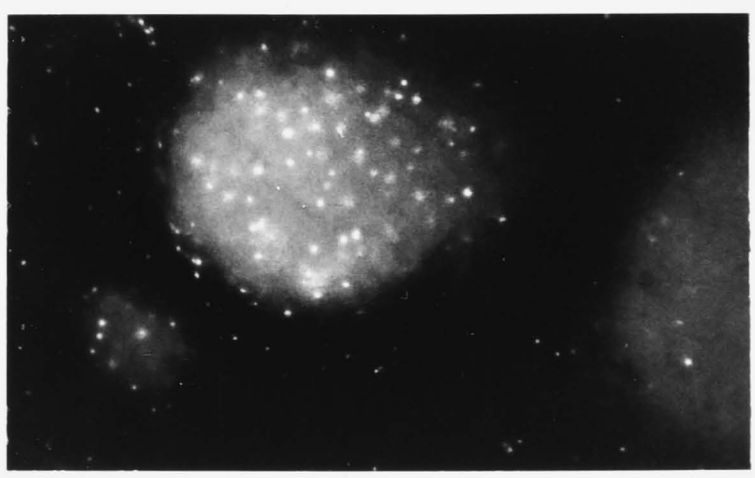


R1+



BCL.1

RD10s



surface molecules (Section 2.8) from different cell lines were bound to a variety of GAGs (chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, endogenous splenic-GAGs and heparin) immobilised on CMC fibres (Section 2.10) and the bound molecules characterised by SDS-PAGE. The results of such an analysis are shown in Figure 6.3 (for all GAGs except heparin) and Figure 6.4 (specifically for heparin-binding molecules) and indicate that a number of molecules on the surface of EL-4, $R1^+$, BCL.1 and $RD10_S$ bind to GAGs.

Each cell line was seen to express distinct GAG-binding molecules with a wide range of molecular masses (Figure 6.3 and 6.4). Heparin-binding molecules (Figure 6.4) were expressed strongly on the cell surface and constitute the entire range of GAG-binding molecules. BCL.1 expressed molecules of approx. 10-22, 35, 47, 53, 120, 172 and 206 kDa with a very prominent GAG-binding molecule at 70-102 kDa which appears to be the equivalent of the 90 kDa GAG-binding protein previously described (Chapter 4). $R1^+$, on the other hand expressed a large range of GAG-binding molecules with molecular masses of 14-22, 27, 30, 35, 38, 45, 53-63, 66 and 90 kDa, and higher molecular mass proteins of approx. 168 and 200 kDa. $RD10_S$ expressed molecules predominantly of intermediate and high molecular masses, namely 58, 65, 102-124 and 150 kDa with a prominent heparin-binding molecule of 35 kDa (Figure 6.4). EL-4 expressed a smaller number of GAG-binding molecules than seen with other cell lines, namely some low molecular mass proteins of 13-22 kDa. However in the heparin-binding profile additional molecules such as the prominent 43-52 kDa band and two bands of 77 kDa and 103 kDa were seen (Figure 6.4). Interestingly, the 90 kDa GAG-binding molecule which was seen so prominently on the surface of splenocytes and thymocytes (Chapter 4 and 5) was only expressed on the surface of the lymphoid cell lines $R1^+$ and BCL.1.

As an interesting side issue, attempts were made to characterise the prominent 43-52 kDa heparin-binding protein on EL-4. Studies have

Figure 6.3 Analysis of ^{125}I-Labelled Lymphoma Cell Surface Molecules Which Bind to Immobilised GAGs

Bound molecules were run on a 8-18% gradient SDS-PAGE gel and gels were autoradiographed for 5 days. Molecular weight markers are indicated in kDa. Ch4S=chondroitin-4-sulfate, Ch6S=chondroitin-6-sulfate, DermS=dermatan sulfate, HepS=heparan sulfate, GAG=endogenous splenic-GAGs.

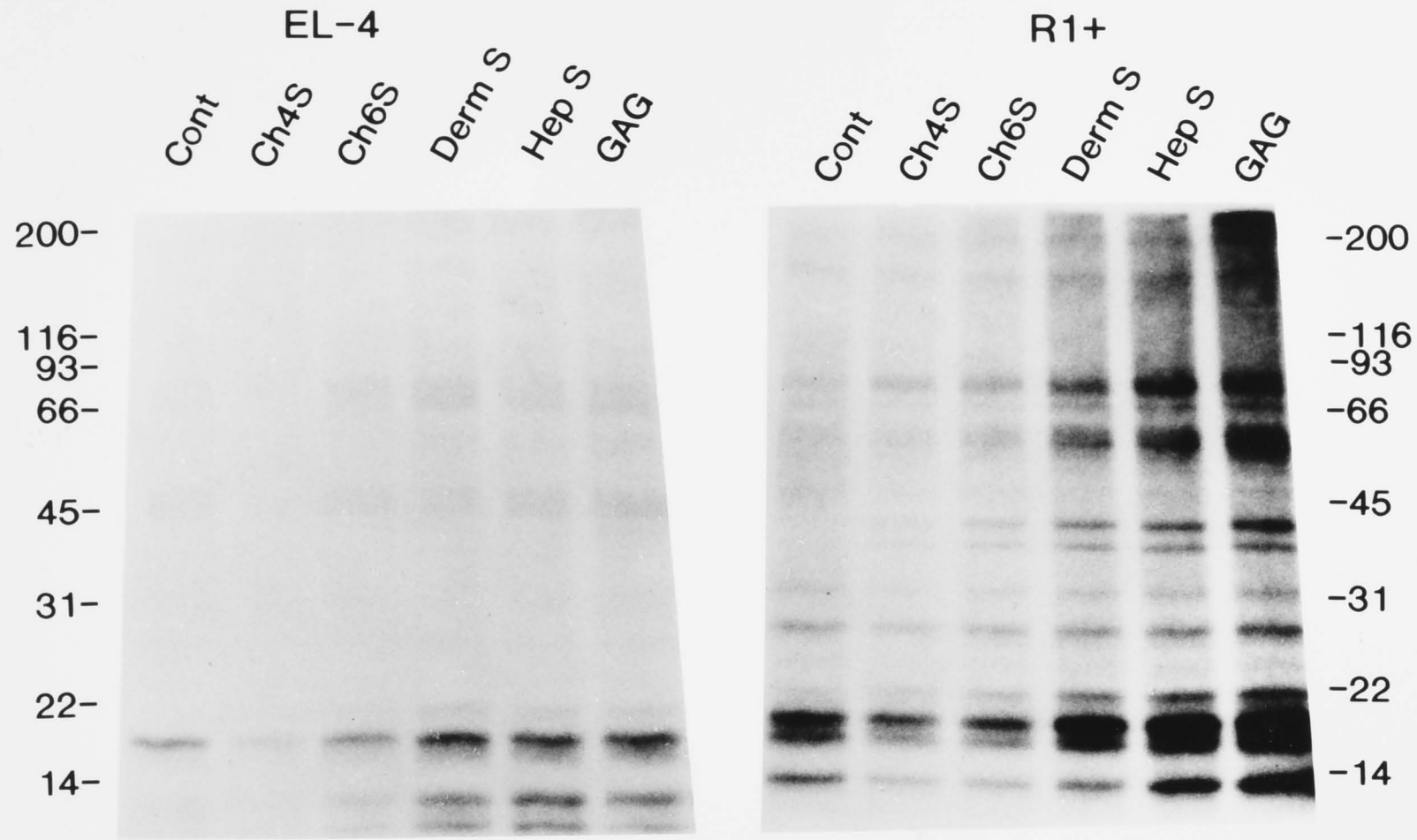
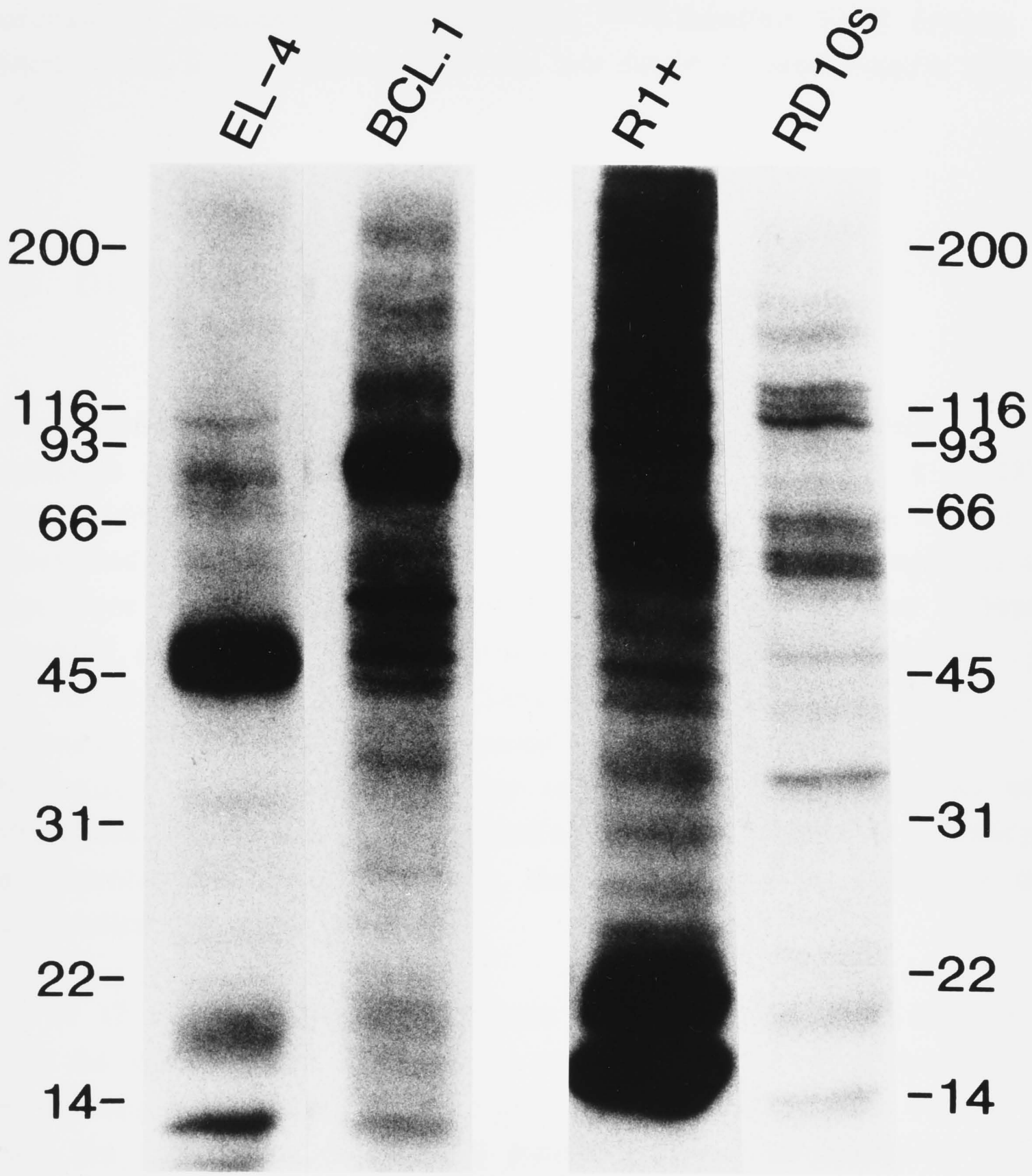

EL-4
Cont
Ch4S
Ch6S
Derm S
Hep S
GAG
200-
116-
93-
66-
45-
31-
22-
14-
R1+
Cont
Ch4S
Ch6S
Derm S
Hep S
GAG
-200
-116
-93
-66
-45
-31
-22
-14
BCL.1
Cont
Ch4S
Ch6S
Derm S
Hep S
GAG
200-
116-
93-
66-
45-
31-
22-
14-
RD10s
Cont
Ch4S
Ch6S
Derm S
Hep S
GAG
-200
-116
-93
-66
-45
-31
-22
-14

Figure 6.4 Analysis of ^{125}I-Labelled Lymphoma Cell Surface Molecules Which Bind to Immobilised Heparin

Bound molecules were run on a 8-18% gradient SDS-PAGE gel and gels were autoradiographed for 2 days. Molecular weight markers are indicated in kDa.


EL-4
BCL.1
R1+
RD10s
200-
116-
93-
66-
45-
31-
22-
14-
-200
-116
-93
-66
-45
-31
-22
-14

shown that a 43 kDa molecule identified by mAb A1 is expressed on the surface of EL-4 and may be involved in binding to carbohydrates (Nagasawa *et al.*, 1987; Yokoyama *et al.*, 1989). This molecule was successfully immunoprecipitated from ^{125}I-labelled EL-4 lysates (Section 2.8, 2.20). However it was not found to bind heparin (Figure 6.5).

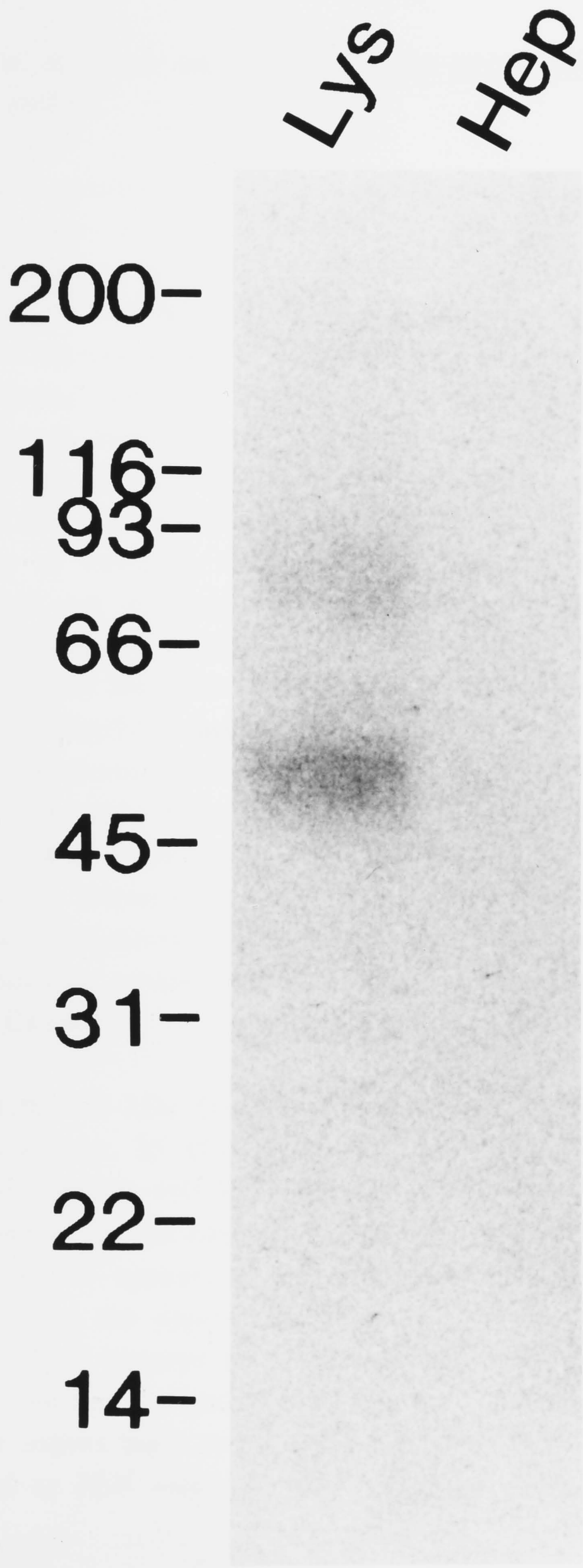
6.3 DISCUSSION

This chapter describes attempts to determine if GAG-binding molecules on the surface of lymphocytes and, in particular, a 90 kDa GAG-binding protein are involved in lymphocyte migration to the spleen and other lymphoid organs. In order to do this lymphoma cell lines were identified which differed in their ability to enter different lymphoid organs and their GAG-binding molecules characterised. It was found that lymphoma cell lines differed markedly in their expression of GAG-binding molecules on their cell surfaces, an observation which confirms earlier receptor studies (Chapter 4) where differences were seen in GAG-binding molecules between splenocytes and thymocytes. It seems likely, therefore, that these molecules may be functionally significant.

Out of 12 cell lines tested for splenic entry, only five were seen to enter the spleen. This may be due to a lack of correct receptors on the cell surface for splenic entry. Alternatively, cell lines may fail to enter the spleen due to physical hindrances such as becoming trapped in other organs including the lungs and liver. This in fact may be the case for some of the cell lines tested, particularly larger cells such as the mastocytoma P815. With this fact in mind, liver and lung entry was determined for each of the four lymphoid cell lines which were studied in detail. It was found that although some cell lines were

Figure 6.5 Ability of the EL-4 Cell Surface Antigen Identified by mAb A1 to Bind to Immobilised Heparin

Anti-EL-4 mAb A1 was used to monitor the presence of radiolabelled antigen in either an unfractionated EL-4 lysate (Lys) or for immunoprecipitation of antigen, ^{125}I-labelled EL-4 molecules eluted from heparin-CMC fibres (Hep). A1 mAb was bound to protein G coupled agarose. Molecular weight markers are indicated in kDa.


Lys
Hep
200
116
93
66
45
31
22
14

trapped in the liver and lungs this did not correlate with splenic-homing ability.

Based on a comparison of the GAG-binding molecules expressed by the different splenic-homing cell lines, the majority of these molecules can be eliminated as being involved in splenic entry. Interestingly, the very prominent 90 kDa GAG-binding protein which was discussed in detail in Chapters 4 and 5 was expressed by two out of four of the splenic-homing cell lines tested. Both BCL.1, a B cell lymphoma maintained *in vivo*, and $R1^+$, a T cell lymphoma propagated *in vitro*, expressed the 90 kDa GAG-binding protein on the cell surface. This indicated that this protein is expressed on a variety of lymphoid cells regardless of culturing methods, an important point considering the molecule is not an integral membrane protein and therefore could be transferred between cells. However, due to the lack of expression of this protein on the splenic-homing cell line $RD10_S$, it is apparent that the 90 kDa GAG-binding protein is not required for splenic entry. Furthermore, homing data presented in Table 6.2 indicates that this protein is probably not required for PLN, PP or MLN entry. Therefore, although the function of this very prominent GAG-binding protein is unknown, an attractive hypothesis is that this molecule is involved in lymphocyte migration events at another level, possibly the positioning of lymphocytes within lymphoid organs. This point will be elaborated upon in Chapter 7.

Although the 90 kDa GAG-binding molecule is not involved in splenic entry, an approx. 35 kDa GAG-binding protein is present on each of the splenic-homing cell lines tested namely, $R1^+$, BCL.1 and $RD10_S$, but is not present on the control cell line EL-4 (Figure 6.3 and 6.4). Furthermore, it appears to be expressed by thymocytes and splenocytes as the approx. 33 kDa GAG-binding protein described in Chapter 4. It remains to be determined whether this protein is directly involved in lymphocyte migration to the spleen or other lymphoid organs such as PP. On the other hand, it does not appear to be related to PLN entry, for $R1^+$ expressed this protein but failed to

migrate to PLN. Further experimentation would be necessary to determine if this protein is present on other lymphoid cell lines that have lymphoid homing potential.

Localisation studies of fluorescently-labelled cells in spleen were employed in an attempt to visualise splenic entry and positioning of lymphoma cell lines. Unfortunately, all three splenic-homing cell lines examined gave a similar localisation pattern, localising in the marginal zones and red pulp and failing to enter the white pulp of the spleen. Previous studies (Brenan and Parish, 1986) suggest that SPS recognition plays a particularly important role in lymphocyte positioning in the white pulp. Nevertheless, there may have been subtle differences in splenic positioning of the lymphoma cell lines which were not detected by the relatively inaccurate fluorescence procedure used. Therefore other methods would need to be used to determine more accurately positioning of cells within lymphoid organs, in order to assess if GAG-binding molecules control positioning of cells within the spleen.

6.4 SUMMARY

This chapter describes attempts to relate the expression of GAG-binding molecules on the surface of lymphoma cell lines to the splenic and lymph node homing capacity of the cell lines.

It was found that certain cell lines failed to enter the spleen (EL-4, BL/VL3, MBL-2, RK4.7, C6VL/1, P815 and LSTRA) while others gained access (BCL.1, $RD10_S$, $R1^+$, $R1^-$ and CL2-FT2). The ability to enter the spleen was not dependent on the cellular origin (T or B cell) or the mode of induction and propagation (*in vivo* or *in vitro*) of the tumour cell lines. Entry of the cell lines into other lymphoid organs, namely PLN, PP and MLN, was found to be unrelated to splenic entry.

Furthermore, PLN and PP entry were not related, although MLN entry appeared to occur as a combination of both PLN and PP entry. Also entry of cells into the liver and lungs did not correlate with splenic entry. Positioning of fluorescently-labelled cell lines in the spleen was visualised by fluorescence microscopy and revealed that certain cell lines ($R1^+$, BCL.1 and $RD10_S$) migrated into the red pulp and marginal zones of the spleen.

SDS-PAGE analysis of ^{125}I-labelled cell surface molecules indicates that each cell line examined (EL-4, $R1^+$, BCL.1 and $RD10_S$) expressed distinct GAG-binding molecules with a wide range of molecular masses. Interestingly the 90 kDa GAG-binding molecule which was seen so prominently on the surface of splenocytes and thymocytes (Chapter 4 and 5) was expressed strongly by both $R1^+$ and BCL.1, but not by $RD10_S$ indicating that this protein is not necessary for splenic entry. However, an approx. 35 kDa GAG-binding protein is present on all three cell lines which migrated to the spleen ($R1^+$, BCL.1 and $RD10_S$) but is absent from EL-4, a cell line which did not enter spleen. Further experiments are required to prove that this molecule is involved in splenic-homing.

CHAPTER 7 : DISCUSSION

One of the major aims of this thesis was to test the hypothesis that recognition of sulfated GAGs plays an important role in the migration and positioning of lymphocytes within the immune system. In the past it has been assumed that the major functional role of SPS and GAGs is to act as a structural component in tissues. For example, hyaluronic acid, the chondroitin sulfates and keratan sulfate are present in large quantities in connective tissues, being essential for their physical properties such as elasticity and compressibility (Obrink, 1975). Also proteoglycans and in particular heparan sulfate proteoglycans can bind together ECM components and mediate the binding of cells to the ECM (Ruoslahti, 1989; Wood *et al.*, 1984, 1985). However, a vast body of data now suggests a more diverse functional role for GAGs which extends beyond them being purely structural elements. This evidence includes the observed structural diversity of GAGs and the ubiquitous presence of SPS recognition systems in many organisms.

Structurally, GAGs are characterised by repeating disaccharide units consisting of alternating uronic acid (glucuronic or iduronic acid) or galactose and hexosamine residues (glucosamine or galactosamine) which in most cases are substituted with sulfate groups. The great variability in GAG structure is the result of variations in monosaccharide constituents, monosaccharide linkages, the degree and position of sulfation, the sequence of disaccharide units and their MW. On this basis alone, heparan sulfate exhibits great structural diversity with an estimated 10^{36} types of heparan sulfate occurring in animal tissues (Dietrich *et al.*, 1983), where heparan sulfate contains a combination of iduronic and glucuronic acid and N-acetyl glucosamine substituted with sulfate groups. Furthermore, not only are GAGs structurally diverse but a wide variety of organ and species specific GAGs also exist (Dietrich *et al.*, 1977; Keller *et al.*, 1978; Toledo and Dietrich, 1977) which suggests that these molecules play more than a purely structural role.

In addition to their structural diversity another important feature of GAGs which may indicate a functional role, is their occurrence in many cell-cell recognition systems (reviewed in Section 1.7). Thus, SPS and GAGs and their complementary lectins have been identified in several cell adhesion systems, such as the reaggregation of marine sponges (Coombe *et al.*, 1987a; Coombe and Parish, 1988), sperm-egg adhesion (Ahuja, 1982; Glabe *et al.*, 1982), neural cell adhesion (Cole *et al.*, 1986; Cole and Glaser, 1986), embryogenesis (Tucker, 1986) and various cell-cell interactions in the immune system (see below).

7.1 GAG RECOGNITION AND LYMPHOCYTE RECIRCULATION

Several lines of evidence suggest that recognition of carbohydrate structures particularly GAGs, is involved in lymphocyte recirculation (reviewed in Section 1.7). For example, the involvement of carbohydrates in lymphocyte homing was demonstrated by the effects of modifiers of carbohydrate structure (Section 1.7.1) and lectins (Section 1.7.2) on lymphocyte migration. Also, lymphocyte/HEV interactions can be inhibited by a range of carbohydrates, including phosphosugars (M6P and PPME) and the SPS fucoidan (Section 1.7.3). Furthermore, SPS receptors have been detected on the surface of lymphocytes (Bradbury and Parish, 1989; Parish *et al.*, 1984, 1988; Parish and Snowden, 1985; Thurn and Underhill, 1986) and certain lymphocyte cell surface antigens, namely Thy-1, CD2, CD4 and CD45 (Ly-5, T200) have been shown to interact with sulfated carbohydrates (Lederman *et al.*, 1989; Parish *et al.*, 1988a, b). In addition, the lymphocyte homing receptors MEL-14 and CD44 may participate in carbohydrate-protein interactions (Coombe and Rider, 1989; Section 1.6.1, 1.7.3).

Direct evidence for the involvement of GAGs in lymphocyte recirculation comes from the finding that certain GAGs cause leucocytosis and inhibit lymphocyte recirculation when injected into animals (Section 1.7.3). However, the most conclusive *in vivo* evidence for GAG involvement in lymphocyte recirculation was the demonstration that GAGs and other SPS selectively effected entry, displacement and positioning of lymphocytes within lymphoid organs (Section 1.7.3; Brenan and Parish, 1986).

In the light of these studies on the involvement of GAGs in the immune system, experiments were undertaken to isolate and characterise endogenous GAGs and lymphocyte receptors for these and other GAGs and determine in part, their role in lymphocyte recirculation *in vivo*.

Several lines of experimental evidence were obtained which suggested that the GAGs present in murine spleen may play a role in lymphocyte migration and positioning. First it was found that endogenous splenic-GAGs isolated from murine spleen bound to murine splenocytes in a saturable, rapid and reversible manner implying the presence of specific receptors on lymphocytes for the endogenous GAGs in spleen (Section 3.2). Furthermore, the predominant endogenous GAG recognised by murine splenocytes was of the heparin/heparan sulfate-like class, the binding affinity for these molecules being very high (K_m of approx. 5 x 10^{-8} M).

Second, of particular interest was the observation that endogenous splenic heparin/heparan sulfate molecules bound to murine splenocytes at a much higher affinity (approx. 20 fold) than bovine lung heparin, suggesting substantial structural specificity in the recognition of this class of GAGs by lymphocytes (Section 3.2; 4.2).

Third, more sensitive detection systems such as rosetting and binding of radiolabelled cell surface molecules to immobilised GAGs demonstrated the presence of cell surface receptors for most classes

of GAGs on at least a subpopulation of lymphocytes (Section 4.2). Furthermore, a great diversity of GAG-binding molecules was detected on the surface of lymphocytes. This diversity was evident from the differences between cell populations (e.g. splenocytes and thymocytes) and lymphoma cell lines (e.g., EL-4, $R1^+$, BCL.1 and $RD10_S$) in the profile of GAG-binding molecules they expressed (Section 4.2; 6.2) and, differences between GAG classes in the molecules they bound (particularly evident with splenocytes).

Fourth, preliminary evidence showing the expression of a 35 kDa GAG-binding protein exclusively on splenic homing cell lines ($R1^+$, BCL.1 and $RD10_S$) suggested that this molecule may be involved in the entry of lymphocytes into the spleen (Section 6.2). However, further work is required to substantiate this observation.

Following the identification of a diverse array of GAG-binding molecules on the surface of lymphocytes, attempts were made to relate these molecules to known cell surface antigens. Unfortunately, immunoprecipitation studies demonstrated that the splenocyte GAG-binding molecules did not correspond to any of the cell surface antigens tested, namely MEL-14, FcR, CD3, ThB, Ly-5, Ly-15, Pgp-1 and Thy-1, although Ly-2 may bind weakly to heparin (Section 4.2.2). The failure of MEL-14 to bind to immobilised GAGs was somewhat surprising as previous studies have shown that this molecule probably interacts with negatively charged sugars such as phosphosugars (M6P, PPME) and SPS (e.g. fucoidan). It could be argued that the incorrect SPS ligands were tested because soluble MEL-14 does not appear to bind to fucoidan and dextran sulfate (Parish, unpublished). Furthermore, evidence suggests that MEL-14 is a calcium-dependent cell adhesion molecule (reviewed by Yednock and Rosen, 1989) and therefore the lack of calcium in these binding studies may have affected the GAG-binding ability of MEL-14.

7.2 IDENTIFICATION OF A NOVEL 90 KDA GAG-BINDING MOLECULE ON LYMPHOCYTES

In the course of these studies a particularly prominent 90 kDa GAG-binding protein was detected on the surface of splenocytes and thymocytes (Chapter 4 and 5). Analysis of lymphoma cell lines which differed in their capacity to enter spleen and lymph nodes demonstrated that cell surface expression of the 90 kDa molecule did not correlate with entry behaviour. Thus, this molecule appears not to control entry of lymphocytes into lymphoid organs.

Although immunoprecipitation studies failed to identify this molecule, experiments were performed to further characterise it for evidence suggested that it was a peripheral rather than an integral membrane protein. Characterisation of this molecule revealed that it is an acidic glycoprotein which is ionically bound to the cell surface. Furthermore, treatment of cells with the integrin-binding peptide, RGDS, failed to release this molecule from the cell surface.

Further characterisation of the 90 kDa GAG-binding molecule suggested that the molecule was bound to the lymphocyte surface via a PI receptor (Section 5.2.2). This was demonstrated by displacement of the 90 kDa protein from the splenocyte surface with inositol hexaphosphate. Furthermore, it was found that other inositol derivatives (inositol hexasulfate, inositol-1-monophosphate and inositol-2-monophosphate) were either ineffective or much less effective than inositol hexaphosphate at displacing the molecule, indicating that inositol hexaphosphate was specifically releasing this protein. The presence of a PI receptor was further confirmed in reassociation studies where the soluble 90 kDa protein was found to reassociate with splenocytes, an interaction which again could be selectively inhibited by inositol hexaphosphate (Section 5.2.3).

An intriguing question is what is the functional significance of the proposed PI receptor on cells? One possibility is that it may allow GPI anchored cell surface proteins to deliver transmembrane signals. In this sense, the PI receptor may explain the ability of certain GPI-anchored proteins (e.g., Qa-2) to deliver transmembrane signals more efficiently than counterparts with membrane spanning polypeptide components (Robinson, 1989). Another possibility is that the PI receptor may aid the internalisation of proteoglycans, the internalised GAG sidechains subsequently regulating cell growth. This appears to be the case with heparan sulfate proteoglycans on hepatocytes where a proportion of the proteoglycans are GPI-anchored to the cell surface and can be internalised via a PI receptor (Ishihara *et al.*, 1987; Ishihara and Conrad, 1989).

Similarly, binding of soluble GAGs to the cell surface via PI-bound receptors (e.g., the 90 kDa protein on lymphocytes) may result in rapid internalisation of the GAGs and modification of cell proliferation. Such a process may be involved in the inhibition of smooth muscle cell growth and cell migration by heparin and heparin-like molecules (Castellot *et al.*, 1981; Majack and Clowes, 1984).

7.3 FUNCTIONAL SIGNIFICANCE OF GAG-BINDING MOLECULES ON LYMPHOCYTES

The question then arises, if most of the GAG-binding molecules on lymphocytes are not intrinsic for splenic entry, what is their functional role in the spleen? One line of evidence suggests that these molecules may be vital for cellular positioning in lymphoid organs. It has been found that injection of certain GAGs such as dextran sulfate into mice radically alters lymphocyte positioning in the spleen (Brenan and Parish, 1986). In fact, lymphocytes were

displaced from the red and white pulp. It seems possible therefore that GAGs may be involved in lymphocyte positioning, while other carbohydrates such as phosphosugars, which are involved in lymphocyte/HEV interactions, may be more directly involved in splenic entry.

Another possible function of GAGs is that they may act in the regulation of cell growth. This may be achieved either directly via GAGs, or indirectly through aiding the action of growth factors. Firstly, as previously mentioned, GAGs have been shown to directly regulate cell growth (reviewed by Ruoslahti, 1989) and may act, in the case of heparan sulfate proteoglycans, following internalisation through a probable PI receptor (Ishihara *et al.*, 1987; Ishihara and Conrad, 1989). Secondly, many growth factors have a high affinity for heparin (Lobb *et al.*, 1986), an interaction which in certain cases can modulate growth factor activity (Imamura and Mitsui, 1987; Neufeld *et al.*, 1987). It is conceivable that heparin-growth factor complexes can bind to GAG receptors on cells, an interaction which may modulate growth factor action. Furthermore, it has even been suggested that heparan sulfate proteoglycan may bind an autocrine growth factor in the ECM and transport it via a PI receptor to the nucleus (Ishihara and Conrad, 1989).

GAG-binding molecules on lymphocytes also could play a role in the process of chemotaxis, lymphocytes being attracted by GAGs released from other cells. Such a process would be important in inflammatory sites, e.g. the release of heparin from degranulating mast cells. Furthermore, GAG recognition could play a role in the entry of lymphocytes into sites of inflammation where GAG receptors could interact with heparan sulfate and chondroitin sulfate proteoglycans exposed on basement membranes (Hassell *et al.*, 1986; McCarthy *et al.*, 1989).

In a more general sense GAG-binding molecules on the cell surface may act as "scavenger" receptors for extracellular GAGs. A possible

example of this is the internalisation and degradation *in vitro* of hyaluronic acid isolated from rat liver endothelial cells (Eriksson *et al.*, 1983). Furthermore, radioactive hyaluronic acid injected into rabbits is taken up to a large extent by the non-hepatocyte cell fraction of liver (Fraser *et al.*, 1981). These findings suggest that hyaluronic acid receptors can mediate endocytosis and subsequent degradation of exogenous hyaluronic acid (Orkin *et al.*, 1982). In this regard, GAG-binding molecules would function in a fairly nonspecific manner, which may not be applicable to the highly specific interactions demonstrated in this study between GAGs and GAG-binding molecules on lymphocytes.

Furthermore, it should be emphasised that it seems unlikely that the GAG-binding molecules detected in this study are interacting with the GAGs in a purely non-specific, ionic fashion. Evidence for specificity includes the finding that charge density alone was not responsible for the inhibition of binding of GAGs to GAG-binding molecules, and minor changes in the sulfation pattern of the polysaccharides had substantial effects on their inhibitory activity (Section 3.2.3).

7.4 FUTURE WORK

This study is seen as the basis for future analyses of the importance of GAGs and GAG-binding molecules in the functioning of the immune system. Obviously there is scope for future research work in this area, as this study has only focused on a small section of the overall system.

The results have indicated that, with the possible exception of a 33-35 kDa GAG-binding protein, no correlation exists between GAG-binding molecules expressed on the surface of lymphocytes and

splenic and lymph node entry. It remains to be determined whether the 33-35 kDa protein is directly involved in lymphocyte migration to the spleen or other lymphoid organs. Investigation of other lymphoma cell lines with splenic-homing ability and ultimately production of mAbs against this molecule may resolve this question.

One possible function of GAG-binding molecules is that they may be involved in lymphocyte positioning. The area of splenocyte positioning *in vivo* and migration of lymphocytes through the spleen is poorly understood. Development of an *in vitro* assay for determining lymphocyte positioning in lymphoid organs, analogous to that developed by Stamper and Woodruff for lymphocyte/HEV binding (1976), would greatly aid experiments in this area. Obviously *in vivo* experiments showing lymphocyte positioning in the spleen following injection of endogenous splenic GAGs would provide one means of determining the role of GAGs in positioning. Furthermore, lymphoma cell lines with unique homing properties for different organs or positions within an individual organ could be used to identify GAG-binding molecules involved in positioning *in vivo*. This approach has only been used in a preliminary sense in this thesis, lymphoma lines with different positioning behaviour in the spleen not being identified. Obviously the isolation of lymphomas from the same progenitor cell line with only minor differences in expression of GAG-binding molecules would be invaluable in determining the effect these molecules have on lymphocyte migration.

Finally, there is great potential for further research into the intriguing anchorage via a possible PI receptor, of the 90 kDa GAG-binding protein to the plasma membrane. The existence of the PI receptor needs to be demonstrated unequivocally. For example, confirmation could be obtained by radiolabelling the PI structure on the 90 kDa protein with ^{3}H-ethanolamine, uptake of labelled ethanolamine by proteins being indicative of a GPI anchor (Low 1989). Furthermore, attempts could be made to displace the 90 kDa

protein from cells with the purified GPI anchor of *Trypanosoma* species (Low, 1989) or by GPI-anchored proteins released from thymocytes by PI-PLC treatment. Also it remains to be determined what other molecules may be attached to the plasma membrane via PI receptors, and are these molecules internalised as proposed herein? Such studies would not only have relevance for lymphoid cells but would have implications for the behaviour of GPI-anchored proteins on all cell types.

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